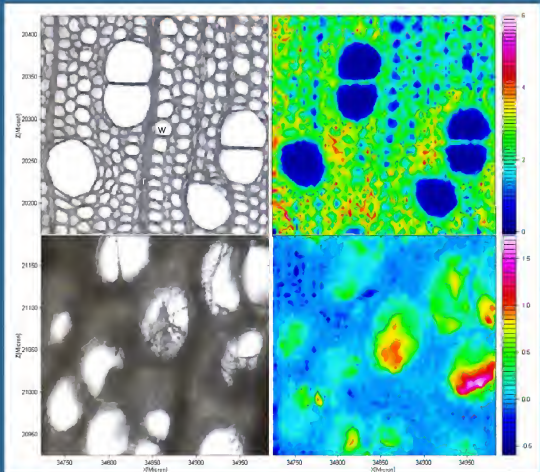


Ursula Kües (Ed.)

Wood Production, Wood Technology, and Biotechnological Impacts



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Footnotes: From 1st of October 2007, institutions of the Faculty of Forest Sciences and Forest Ecology at the Georg-August-University of Göttingen have been fused into two larger institutes and accordingly renamed

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Description of Cover Photos

Cover illustrations

The cover photos are illustrations to Chapter 9 (A. Naumann, S. Peddireddi, U. Kües & A. Polle: Fourier Infrared Microscopy in Wood Analysis) and Chapter 6 (T. Teichmann, W.H. Bolu & A. Polle: Transgenic Trees).

Fig. on the Front Cover

FTIR (Fourier Infrared Microscopy) spectra of beech wood and beech wood infected with *Schizophyllum commune*. Top Panel. Uninfected beech wood as seen in the light microscope (left) and false colour image of the same beech wood section measured with a MCT (mercury cadmium telluride) single channel detector of the FTIR microscope (right). Bottom Panel. Infected beech wood as seen in the light microscope (left) and false colour image of the same beech wood section measured with a MCT single channel detector of the FTIR microscope (right) (for further explanations, see Chapter 9 of this book).

Fig. on the Back Cover

Top. Wood from non-transformed poplar. Bottom. Wood from antisense *CAD* poplar showing a red colour due to an increased content of cinnamyl aldehydes in the lignin modified by genetic engineering (for further explanations, see Chapter 9 of this book)

Part I – Prologue to this Book

1. Molecular Wood Biotechnology – Defining a New Field of Research

Ursula Kües

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Introduction

The Deutsche Bundesstiftung Umwelt - shortly DBU (Fig. 1) - was established by the German state in 1991 with a domicile in Osnabrück. The DBU is a governmental foundation to support innovative exemplary projects in the field of environmental protection and it is the biggest of its kind in Europe (<http://www.dbu.de/stiftung/>).



Fig. 1 Logo of the Deutsche Bundesstiftung Umwelt

A special programme of the DBU was the institution of university chairs ("Stiftungsprofessuren") for trendsetting environmental tasks. Upon a call to German universities for an endowment of such a new chair, the Faculty of Forest Sciences and Forest Ecology at the Georg-August-University Göttingen was awarded in the year 1999 a chair for "Molecular Wood Biotechnology". The reasoning behind the decision of the DBU was as follows in citation of an announcement in German language (<http://www.dbu.de/press/artikel283.html>) and then in translation:

"Holz ist ein nachwachsender Rohstoff, der in umweltverträglicher Weise nachhaltig produziert und genutzt werden kann. Obwohl die große potenzielle Bedeutung von Holz als umweltverträglicher Rohstoff erkannt ist, werden die Einsatzmöglichkeiten bei weitem noch nicht ausgeschöpft. Durch die Stiftungsprofessur sollen Verfahren für die Verwertung von Holz erforscht und die Grundlagen für neue Produkte aus Holz entwickelt werden, die sich am Leitbild einer nachhaltigen multifunktionalen Waldbewirtschaftung orientieren." In translation: "Wood is a renewable resource that can be produced and used in a sustainable manner. Although the huge potential of wood as environmentally friendly material is well recognised, the possibilities for applications are yet by far not exploited. New processes for wood utilisation shall be investigated and fundamentals for new wood products be developed by the founded chair ("Stiftungsprofessur") under the overall concept of a sustainable multi-functional forest utilisation."

Guidelines for the chair

Currently, the renewable resource wood is used mainly as timber in constructions (indoors and outdoors), in furniture industry, in wood composite production (plywood, particleboards, fibreboards, oriented-strand-boards, and plastic-wood-composites) and in pulp and paper industry (see Chapter 2 of this book). In many instances, the desired application of wood necessitates relative extensive pre-treatments and physical and chemical processing (for examples, see Chapters 13, 15, 16, 18, and 20 of this book). Often, these processes are incriminatory to the environment and highly energy-consuming (some keywords: kraft pulping liquors, spent liquors from sulphite pulping, preservatives, etc.) and in the current pulping procedures, the full potential of the resource wood is not exploited.

Biotechnological processes are so far seldom applied in the wood industry, i.e. the application of living organisms or of biological systems in the production and conversion of materials. However, manifold possibilities exist in modern biotechnology to extend utilisation of wood and wood products and to contribute to the development of environmentally friendly production processes and products (Mai et al. 2004a; this book). The chair "Molecular Wood Biotechnology" at the Faculty of Forest Sciences and Forest Ecology at the Georg-August-University Göttingen is implemented by the DBU under the direction to expand the range of biotechnological applications on wood. Particular tasks are to broaden the possibilities of utilisation of the resource wood by application of molecular biological techniques,

to investigate molecular biological processes for new applications of wood and to invent new, environmentally friendly products within the frame of a sustainable, multi-functional forest utilisation. Next to a strong application of modern molecular biological methods in biotechnology, cooperations with related research fields in university and with practical industry are anticipated and the installation of an interface between research and teaching. In teaching, input is requested in the forestry bachelor education (<http://www.forst.uni-goettingen.de/studium/bachelor/einfuehrung.shtml>) and in the master programmes “Wood Biology and Wood Technology” (<http://www.forst.uni-goettingen.de/studium/hb/einfuehrung.shtml>) and “International Forestry” (<http://www.forst.uni-goettingen.de/studium/tif/intro.shtml>).

The potential fields of research cover a wide variety of topics. These include (i) genetic engineering of trees, (ii) implication of biological wood protection methods, (iii) environmentally sound manufacture of wood products, (iv) recycling of refuse from wood industry and used wood and wood products and (v) biotechnological waste management of liquid and solid substances from the wood industry and from wood and wood products during and after use. The person appointed to the chair had the freedom to choose from this wide variety of topics suitable fields and focal points of research.

Own fields of research

The own research field at the time of appointment to the chair “Molecular Wood Biotechnology” at the Faculty of Forest Sciences and Forest Ecology of the Georg-August-University Göttingen was purely in basic research and focused since 1990 for ten years on developmental processes of the basidiomycete model fungus *Coprinopsis cinerea*, at that time called *Coprinus cinereus* (Kües 2000). White and brown rot fungi, groups of organisms responsible for wood degradation, are mostly basidiomycetes (Schwarze et al. 2000; see Chapter 17 of this book). Research projects on lignocellulose-degrading fungi and their products (enzymes, metabolites, fruiting bodies) fall into the frame of research topics anticipated for the chair. Fungal products might be used as tools in industry for improving and altering wood properties, in manufacture of wood products and in waste management and recycling. In contrast, fungal products can also be obtained from wood by conversion of biomass. The idea was therefore to broaden the research on the inkcap fungus *C. cinerea* into applied fields and to extend the species range of studies to various white-rot fungi such as *Heterobasidion annosum*, *Pleurotus ostreatus*, *Schizophyllum commune*, *Trametes versicolor* and other Coprinoids (Rühl et al. 2007).

Basic and applied research

Having studied biology at the Ruhr-University Bochum with a focus on botany, the practical diploma thesis (Kües 1983) dealt with the Mendelian genetic back-

ground on flocculation (spontaneous aggregation of single cells in suspension) of the baker's yeast *Saccharomyces cerevisiae*. Obviously being very academic, this subject has however also a strong practical background for industrial applications. For example, timely onset of flocculation enables the cheap and easy separation of yeast cells from beer directly after completing the brewing process and flocculation of micro-organisms is an integral element in the activated sludge processes in waste water treatment (Esser & Kües 1983). Evaluation of the chemical nature of the yeast flocculation mechanism and invention of a method for deflocculation (Stahl et al. 1983) during the diploma work subsequently allowed in industrial fermentations to manipulate the onset and degree of yeast flocculation according to process need (Esser et al. 1987, Masy et al. 1991, Soares & Seynaeve 2000).

The following PhD work (Kües 1988) concentrated on elucidating replication and recombination of bacterial plasmids (extrachromosomal DNA that is not essential for survival of the organisms), i.e. how these DNA molecules propagate in cell proliferation and how they rearrange in their genetic structures (Kües & Stahl 1989, 1992, Kües et al. 1989). Again, principle questions of academic concern were addressed whilst the work was initiated by an industrial interest on the methanol-consuming bacterium *Methylomonas clara*. In the 1970s with the increasing worldwide human population, this methylotrophic bacterium was once considered as an alternative source of high-quality-food ("single-cell-protein") produced in fermenters from methanol as an industrial waste (Faust et al. 1977). However, such single-cell-protein production became unattractive by increasing petroleum prizes and with the awareness that crude oil is a restricted resource (Puhar et al. 1982, deVries et al. 1990). Whilst a sophisticated fermentation technology existed for the bacterium (Liefke & Onken 1992), new applications were sought for *M. clara* by genetic engineering for recombinant protein production. To this end, vectors for gene cloning and DNA transfer were developed from natural narrow-host-range plasmids of *M. clara* and genetic methods for the DNA transfer were established for this biotechnological interesting bacterium that formerly was not accessible to genetic engineering (Kües & Stahl 1992). The position of recombinant protein production by methylotrophic organisms was however quickly overtaken by the easier to handle, more versatile yeasts *Hansenula polymorpha* and *Pichia pastoris* (Gregg et al. 1993, Porro et al. 2005; see Chapter 19 of this book), but methylotrophic bacteria still have their place in production of specific amino acids (Gomes & Kumar 2005).

In a first post-doctoral position, academic studies on genes conferring heavy-metal resistances to bacteria (Dressler et al. 1991) lead to the construction of genetically engineered yeast cells for enzymatic detoxification of mercury salts (Rensing et al. 1992). The principle concept was later on successfully transferred to plants including trees for phytoremediation of mercury salt-contaminated soils (see Chapter 7 of this book).

To newly combine genes from a genetic pool of eukaryotic organisms in nature, sexual reproduction is required and at least two different sexes (male and female) - but also not more than two. Accordingly, this is the normal common situation for most eukaryotic species. It is however not fully understood why it is usually so (Whitfield 2004). Biologists have puzzled about this “two sexes”-phenomenon ever since the father of theoretical biology, R.A. Fischer, addressed this scientific-philosophical question and suggested to study species that break the two sexes-rule by having more (Fischer 1930). Interestingly, most basidiomycetes very much exceed the number of two sexes in up to ten thousands per species. For example, the model species *C. cinerea* has more than 12.000 (Kües 2002). Technical, sexes in the fungi are termed mating types since they base not on morphological differences but on physiological self-incompatibilities (Kües & Casselton 1992). Genetically, they are therefore comparable to the multiple self-incompatibility mechanisms of plants superimposed on the male and female sex-organs (Hiscock et al. 1996, Charlesworth et al. 2005). The multiple fungal and plant self-incompatibility systems promote a higher degree of outbreeding (mixing within the genetic pools) than simple systems with two different sexes - which is a plausible reason for the development of multiple systems in contrast to the more general phenomenon of only two sexes (Hiscock & Kües 1999, Kües 2002). The choice of outbreeding *versus* inbreeding influences gene flow within populations and thus the variability and fitness within species and their short-term and long-term adaptations to the changing environment (Charlesworth 2003, Neiman & Linksvayer 2006; see also Chapter 8 of this book). Research in the last decade lead to the identification of the cellular gene functions defining the mating types in *C. cinerea* and other basidiomycete and, thereby, controlling sexual development (Casselton & Olesnický 1998, Hiscock & Kües 1999, Kües et al. 2001, Kües 2000, James et al. 2006, Srivilai et al. 2006b, Casselton & Kües 2007). One might expect that there is no direct link between the academic interest in evolution of sexes and mating types and any problem in biotechnology. However, the fungal mating type genes control fruiting body development (Kües et al. 1998, 2002b, James et al. 2006) and are therefore of importance for the production of edible mushrooms, as well as for breeding programmes of better production strains (Kües and Liu 2000, Kothe 2001; see Chapters 22 and 23 of this book). Edible and medicinal mushrooms have a well-balanced nutritional composition and contain various substances with (potential) positive effects on human health and well-being (Wasser 2002, Kües et al. 2004, Zaidman et al. 2005). In many instances, such mushrooms are cultivated on straw, wood and wood wastes, giving a direct link to the field “Molecular Wood Biotechnology” (see Chapter 22 of this book).

All research examples from the past show that basic and applied research go hand in hand and profit from each other, regardless of whether basic research stand at the beginning as in the studies of yeast flocculation, fungal mating types and fruiting body development, or whether an applied problem initiated the

research as in the case of the studies of bacterial heavy metal resistance genes provoked by industrial soil pollution and in the case of the studies of the *M. clara* plasmids by the purpose of genetic engineering of the bacterium.

Accordingly, basic and applied questions are both legitimate reasons for research that ultimately targets at environmentally friendly applications in the forestry and wood sector as requested by the DBU and by the Faculty of Forest Sciences and Forest Ecology of the Georg-August-University Göttingen to be performed in frame of the chair “Molecular Wood Biotechnology”.

Research in the laboratory of “Molecular Wood Biotechnology”

Starting from scratch in an innovative field newly to be defined, it appeared best to take suitable tools and knowledge from former research work. The model species *C. cinerea* is accessible by both Mendelian and molecular genetics (Granado et al. 1997, Kües et al. 2001b, Walser et al. 2001) and by an available genome sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) that is in the state of gene annotation (Kües et al. 2005). This best established system amongst higher basidiomycetes was further developed so that we know have an overexpression system for enzymes and other proteins at hand that we use for example for the production of laccases (Kilaru et al. 2006b,c; see Chapter 19 of this book). Moreover, as exemplified with *C. cinerea* laccase Lcc1, we can target expression specifically to the mushrooms (Velagapudi 2006, Velagapudi et al. 2006).

Laccases are enzymes able to attack lignin and this property can be exploited in environmentally friendly production of wood composites (Hüttermann et al. 2001, Mai et al. 2004a; see Chapters 17 and 18 of this book, Fig. 2). Former work



Fig. 2 Wood of low quality can be chipped or disintegrated into fibres, the chips and fibres incubated with a laccase solution (present in the Erlenmeyer flask) to activate the natural bonding forces of lignin in order to subsequently be pressed into particleboards (shown at the left) or medium-dense-fibreboards (MDF), respectively. Further details are given in Chapter 18 of this book. The photo was kindly supplied by C. Schöpfer

identified three different laccase genes in a genomic library of *C. cinerea* (Bottoli et al. 1999). Additional five were identified by experimental work in the laboratory in Göttingen (Hoegger et al. 2004) and, after release of the genomic sequence of the fungus by the Broad Institute (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/), nine more by genome analysis with computer programmes (Hoegger et al. 2006a, Kilaru et al. 2006a). Vector constructs for overexpression of all these enzymes in *C. cinerea* as well as in the yeast *S. cerevisiae* are available (Kilaru 2006, Kilaru et al. 2006b,c) and we started to biochemically characterise the various enzymes (Saathoff 2005, Kilaru 2006, Kilaru et al. 2006c, Kellner et al. 2007, M. Rühl et al. unpublished). Similarly, we are exploiting the *C. cinerea* overexpression system for enzymes from other fungi (Grimrath 2007; P.J. Hoegger and S. Kilaru, unpublished). First experiments with recombinantly produced enzymes in fibreboard production have been performed and also with enzymes obtained from wastes from mushroom production (Rühl et al. 2005a,b, 2006, Fischer 2006). Other current applications of laccases target at dye decolourisation (Svobodová et al. 2003, 2008, Svobodová 2005, Kilaru 2006). Moreover, amongst various other applications (Kilaru 2006; see Chapters 17 and 19 of this book), laccases are very suitable to develop devices for detection of toxic phenolic compounds in various kinds of material and environment (see Chapter 12 of this book).

Some fungi have the ability to enzymatically degrade phenolic and other toxic compounds and are therefore useful for bioremediation (Mai et al. 2002, 2004a,b, Majcherczyk et al. 2003, Eggen and Majcherczyk 2006; see Chapter 17 of this book). Genomics and proteomics are used to identify new enzymes with new properties from *C. cinerea* and various white-rot fungi for biotechnological applications in the wood industry (Dwivedi 2006, Dwivedi et al. 2006, Hoegger et al. 2006a, Kilaru 2006, Kilaru et al. 2006a). Actual enzyme properties will define the type of application new enzymes might be used for.

By its complexity, wood degradation by white-rot fungi is still only poorly understood (Leonowicz et al. 1999, 2001, Messner et al. 2003, Martínez et al. 2005; see Chapter 17 of this book). Fungal attack of wood is followed under microscopes to detect fungi and fungal damage in wood, using newly developed methods such as FTIR (Fourier transform infrared microscopy) and new stains reacting with wood degradation products (Lang 2004, Naumann et al. 2005, Korus 2006; see Chapter 10 this book). Different fungal species within wood were possible to distinguish by FTIR (Naumann et al. 2005). Early detection of fungi in wood by such methods as well as by means of DNA isolation and characterisation (Hoegger et al. 2006b, Naumann et al. 2007; see Chapters 9 and 10 of this book) is of importance for rescuing wood and wood products by taking appropriate measures against the infecting fungi. Moreover, from studying lignocellulose degradation by fungi in wood and straw one can expect to also identify further enzymes with interesting properties (Dwivedi 2006; see Chapter 17 of this book). Experiences with wood degradation by fungi will also give new inputs in composting expe-

riments for environmentally friendly disposal of used and non-recyclable wood products and for defining wood products of better durability. Quality control is therefore also an issue of research and development (Hoegger et al. 2007; see Chapter 15 of this book). Effects of toxic volatile organic compounds (VOCs) from wood and wood products can harm or, at higher concentration, even kill eukaryotic cells as exemplified with cells of the yeast *S. cerevisiae* (Schützendübel et al. 2005, Pemmasani 2006; see Chapter 12 of this book). Suitable methods for detection of harmful VOCs have to be identified and optimised (Pemmasani 2006; see Chapters 11 and 12 of this book).

Mushroom development is another strong area of research in the laboratory (Liu et al. 2006, Navarro-González et al. 2006, Srivilai 2006, Srivilai et al. 2006a; see Chapter 23 of this book). From our Thai colleagues, we newly learned that *C. cinerea* is produced for human consumption in Thailand by many small family businesses for sale on local markets as a fresh mushroom or a pickled delicacy known as “Hed-Cone-Noy”, in translation “Small Compost Fungus” (Tapingkai 2004, W. Chaisaena & P. Srivilai, personal communication). The fungus is thus not only a genetic model for basidiomycetes but has also a broader implication for humans. As other mushrooms, the species produces various odours (Thakeow et al. 2006; see Chapter 11 of this book) and it contains substances of medical interests (Badalyan et al. 2005a,b). Amongst are small secreted sugar binding proteins known as galectins (Boulianne et al. 2000, Walser et al. 2003, 2004, 2005) that, by analogy of a highly similar galectin from the mushroom *Agaricus aegerita* (Hizukuri et al. 2005, Yang et al. 2005), could have anticancer effects. Mushrooms as well as the fungal mycelium produce another interesting class of small secreted proteins with potential in medical and technical applications, the hydrophobins that are characterised by formation of amphiphatic protein films (Walser et al. 2003, Scholtmeijer et al. 2001, 2004, Velagapudi 2006). *C. cinerea* as well as the white-rot fungus *Phanerochaete chrysosporium* and the ectomycorrhizal species *Laccaria bicolor* have large families of hydrophobin genes awaiting their biotechnological exploitation (Velagapudi et al. 2005a,b, Peddireddi et al. 2006, Velagapudi 2006).

In conclusion, the general concept to define the research field “Molecular Wood Biotechnology” moved from basic research of a single fungus, *C. cinerea*, into applied research with various white-rot fungi. Current foci are on fungal enzymes involved in wood degradation - their identification, production and application, on identification techniques of fungi in wood, on environmentally friendly production of wood composites, on quality control of wood and wood products in service, on biological disposal of wood products after service, and on mushrooms as non-timber products from straw and wood. Evolutionary and ecological aspects of diversity of fungal enzymes and other proteins are also addressed (James et al. 2002, 2004, 2006, Kilaru & Kües 2005, Pemmasani et al. 2005, Hoegger et al. 2006a, Kilaru 2006, Kilaru et al. 2006a, Kües et al. 2006, Velagapudi 2006) and we study growth of basidiomycetes on straw, wood and other plant

litter (Badalyan et al. 2003a,b, Badalyan & Kües 2004, Sánchez Hernández 2007), vegetative and sexual reproduction of these group of fungi (Polak et al. 2001, Kües et al. 2002a, 2004, Lu et al. 2003, Badalyan et al. 2004, Domingo-Martínez 2005, Fischer & Kües 2003, 2006, James et al. 2006, Liu et al. 2006), and interactions with other organisms including animals (Navarro-González et al. 2007).

The NHN

Technology transfer of results from the laboratory to industrial application requires interactions with industry. To this end, the “Kompetenznetz für Nachhaltige Holzforschung” (NHN; Competence Net for Sustainable Wood Utilisation; in the three-year beginning phase known as “Niedersächsisches Kompetenznetz für Nachhaltige Holzforschung”; <http://www.kompetenznetz-holz.de/>; Fig. 3) was founded in Göttingen between the Georg-August-University Göttingen (founding members: Prof. Dr. A. Hüttermann, Prof. Dr. A. Kharazipour, Prof. Dr. U. Kües and Prof. Dr. A. Polle, all Institute of Forest Botany, and Prof. Dr. H. Militz, Institute of Wood Biology and Wood Technology; later entry: Prof. Dr. B. Möhring, Institute of Forest Economics), the Fachhochschule Hildesheim/Holzminde/Göttingen (founding members: Prof. Dr. F. Bombosch, Fakultät für Ressourcenmanagement, Prof. Dr. W. Viöl, Fakultät für Naturwissenschaften und Technik), the Niedersächsische Landesversuchsanstalt (now the Nordwestdeutsche Forstliche Versuchsanstalt) in Göttingen (founding member: Prof. Dr. H. Spellmann) and the Wilhelm-Klauditz-Institut Braunschweig (WKI, Fraunhofer Institut für Holzforschung; founding member: Prof. Dr. R. Marutzky) and the Technical University of Braunschweig (later entry: Dr. Ing. H.-W. Hoffmeister, Institut für Werkzeugmaschinen und Fertigungstechniken, IWF) and partners from the industry.



Fig. 3 Logo of the NHN

Kompetenznetz für Nachhaltige Holznutzung

With the different partners, the NHN represents the whole forest-wood-chain. For the first time in Germany, it brings together such different expertise as found in forest management, in wood technology and industry, in biotechnology and molecular biology, in mechanical engineering, and in economy. The overall activities of the NHN divide in Research and Development (R & D) and Information and Marketing (I & M). Main research areas include ecological and market-oriented forest management (for background reading see Chapters 2 to 6 of this book),

modern logistic and effective concepts for wood sorting, production of innovative wood composites (see Chapters 15, 16, 18, and 20 of this book), biotechnological applications in the wood industry (for examples see Chapters 7, 14, 18, 21, 22, and 24 of this book), wood modification, surface treatment and preservation (see Chapters 13 and 14 of this book), and product and process analysis (see Chapter 15 of this book). A coordinator (until April 2006: B. Rüther) organises the business side and the interactions between the partners. More about the different partners, the activities and research projects of the NHN can be found on the NHN homepage (<http://www.kompetenznetz-holz.de/>). The NHN has been evaluated and admitted as a member in [kompetenz-netze.de](http://www.kompetenz-netze.de), an initiative of the German Governmental Ministry for Education and Research (Bundesministerium für Bildung und Forschung, BMBF) for support of the international marketing position of highest-capacity competence networks of Germany (<http://www.bmbf.de/pub/innovationsfoerderung.pdf>; Federal Ministry of Economics and Technology 2006).

The NHN started in 2002 with a three year financial support from the Ministry of Culture and Education of Lower Saxony (project leader: A. Polle, speaker of the executive board of the NHN till October 2005) and from the European Regional Regional Development Fund (“Europäischer Fonds für regionale Entwicklung”, EFRE; project leader: U. Kües). Due to this financial support and additional support by the Pfeleiderer AG, Neumarkt, Germany, a laboratory for wood technologies, the “Biotechnicum”, was build in Göttingen on the grounds of the Georg-August-University at the Institute of Forest Botany and equipped amongst other machinery with pilot plants for wood composite production and modern machines for testing wood properties (see Chapter 15 of this book). Furthermore, fermenters up to a volume of 500 l are available for low and high-scale production of fungal enzymes for use in wood modifications (see Chapter 19 of this book). The funding by the MWK and by EFRE enabled to start projects to develop new generations of wood composites (see Chapter, 15, 16, and 18 of this book) and innovative methods for wood and wood product characterisation by FTIR microscopy and molecular biology (see Chapters 9 and 10 of this book).

Interactions between NHN partners from research institutes and partners from the industry enabled by now to acquire some innovative projects for developing and testing new, environmentally friendly wood composite products and insulating materials from fast growing tree species up to the industrial scale (Anonymous 2005, 2006). After the three years start-up phase, the NHN has become a well-known and strong establishment for applied research in the wood sector in Germany (Rüther 2005). Support by EFRE intended the foundation of a spin-off from the competence-net NHN at the end of the funding period in autumn 2005. On the 10th of October 2005, an incorporated association NHN was launched (Rüther 2005). This incorporated association has the tasks to act as a platform to bring together researchers and industrial partners from different fields for a most

efficient cooperation in developing and finding new solutions to problems arising around the resource wood, and to coordinate research and development projects between different partners (<http://www.kompetenznetz-holz.de/>). In addition, funding by EFRE for I & M resulted in the spin-off of the Office for online-Communication "Mut online" by D. Melle and S. Thomas (<http://www.mutonline.de/>).

Integration within the Faculty of Forest Sciences and Forest Ecology

Within the teaching programmes of the Faculty of Forest Sciences and Forest Ecology (Fig. 4) at the Georg-August-University Göttingen, the study topic "Molecular Wood Biotechnology" is integrated in the master and the international PhD programmes "Wood Biology and Wood Technology" (master programme: <http://www.forst.uni-goettingen.de/studium/hb/einfuehrung.shtml>; PhD programme: <http://www.wood.uni-goettingen.de/phd/>). Teaching focuses on general biotechnology and, more specifically, on biotechnology in wood biology and wood industry, but also on tasks of biotechnology, microbiology and molecular biology in preserving and improving the environment. Research directions in the field "Molecular Wood Biotechnology" and major results from own research are included in teaching to necessity. Master students and PhD students are introduced into the practical sides of biotechnology and molecular biology in laboratory courses on different micro-organisms, DNA, RNA and proteins. Practical knowledge is further mediated in bachelor, master and PhD thesis work. In addition, teaching for bachelor students is performed in forest botany and forest pathology, and for master students of all master programmes of the Faculty of Forest Sciences and Forest Ecology (<http://www.forst.uni-goettingen.de/studium/master/einfuehrung.shtml>) and interested students of other faculties of the Georg-August-University Göttingen in form of lecturing and practical courses in forest pathology and in fungal ecophysiology and genetics.

Common teaching courses with other professors of the Faculty of Forest Sciences and Forest Ecology (Prof. Dr. A. Polle: Forest Botany and Tree Physiology; Prof. Dr. R. Finkeldey: Forest Genetics and Tree Breeding;



Fig. 4 Logo of the Faculty Forest Sciences and Forest Ecology at the Georg-August-University Göttingen

Prof. Dr. S. Schütz: Forest Zoology and Forest Conservation; Prof. Dr. H. Militz: Wood Biology and Wood Technology) reflect overlaps in research interests. Accordingly, common student and research projects have been initiated and already a few common papers (Kües et al. 2003, Mai et al. 2004a, Naumann et al. 2005, Peddireddi et al. 2006, Blödner et al. 2007, Chapters 10, 12, 14, 15, 18 and 24 of this book) and abstracts (as examples see Schützendübel et al. 2005, Velagapudi et al. 2005a,b, Thakeow et al. 2006, Navarro-González et al. 2007) been published. The NHN represents another strong link in research within and above the borders of the Faculty of Forest Sciences and Forest Ecology (see above).

This book

At the end of the funding period of the chair “Molecular Wood Biotechnology” by the DBU, this book presents to the public an overview on the scientific activities of the laboratory since its foundation and is intended as an acknowledgment to the DBU for its generous support. Contributors to this book are colleagues and coworkers from the Institute of Forest Botany, from the Faculty of Forest Sciences and Forest Ecology and from the NHN. To all authors, I would like to address my special gratitude for supporting me in production of this timely book on “Wood Production, Wood Technology and Impacts by Biotechnology”. Currently, the book is unique in its kind and gives overviews on various subjects that have not been reviewed before. In other fields, the chapters provide the up-to-date knowledge. I hope this book closes a textbook gap for devoted master and PhD students of “Wood Biology and Wood Technology” and gives scientists compact summaries on specific research subjects in the field.

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Part II – Wood as a Resource

2. The Wood Supply in the World, Europe, Germany, and Lower Saxony

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Introduction

The world's forest areas are decreasing in an alarming manner whilst the wood consumption of the world is increasing enormously due to the exponential growth of the world population and the rising prosperity in several continents. Worldwide shortages of wood resources are looming up, in spite of an increase of forest plantations and of rising wood production (FAO 1999; see also Chapters 3 and 4 of this book).

The Global Forest Resources Assessment 2005 estimates the total area of the world's forests to be just under 4 billion hectares (FAO 2005a). The deforestation amounts to about 13 million hectares per year (2000-2005). At the same time, there is an annual increase of 5.7 million hectares of forest area as a result of forest planting, landscape restoration and natural expansion of forests. The rate of

deforestation seems to slow down lately. Nevertheless, the net annual loss of 7.3 million hectares in the period 2000-2005 is still considerable (FAO 2005b). The major net losses occurred in South America and Africa, where the forest area decreased by 4.3 and 4.0 million hectares per year, respectively, whereas Asia developed to a region with a net gain of one million hectares per year in the same period (FAO 2005c, further information in Chapter 4 of this book).

In 1996, the world-wide total wood supply run up to 3.35 billion m³, of which 1.62 billion m³ had been felled in the tropics. Four fifth of the tropical fellings had been used as firewood. From the remaining 1/5 – or 290 million m³ – only 70.9 million m³ or 24% had been exported. Main importing countries were China, Japan, and other Asian countries. In 2001, only about 1.84 million m³ of timber and timber products have been imported by Germany. However, the trend is decreasing according to time progression (Dieter 2003).

Wood supply in Europe

The total forest area of Europe was just above 1 billion hectares in 2005, which is slightly more than one quarter of the global forest area and 44% of the total land area in Europe. More than 80% of Europe's forests are growing in the Russian Federation. Another 11% of forest area are located in Sweden, Finland, France, Spain, Germany, and Turkey. About 85% of Europe's forests are available for wood supply (MCPFE & UNECE/FAO 2003). Between 2000 and 2005, the area of Europe's forest expanded by about 660,000 hectares per year, although at a slower rate compared with the 1990s (FAO 2005c).

In Europe, the annual increment of forests and other wooded land amounts to 2,287 million m³. The annual increment is at the highest level ever known and exceeds by far the annual fellings of 627 million m³ (over bark). The wood utilisation rate (ratio of the annual fellings to the annual increment) is only 27%. Most countries lie above this average (MCPFE & UNECE/FAO 2003; see Fig. 1), which is mainly caused by the very low utilisation rate of the forests of the Russian Federation, not even reaching 10%.

The total wood produced in Europe of logs, fuelwood and pulpwood averages about 444 million m³ annually over the last past years (MCPFE & UNECE/FAO 2003). This is 13% of the World's total wood supply.

The European Timber Trend Study V (UNECE/FAO 1996) predicted for 18 Western European countries an increase in production of forest products as well as in wood consumption, between 1990 and 2020. In spite of the risk of depletion in some regions, Europe will be able to meet the challenge of increasing wood demand without threatening the sustainability of its forests.

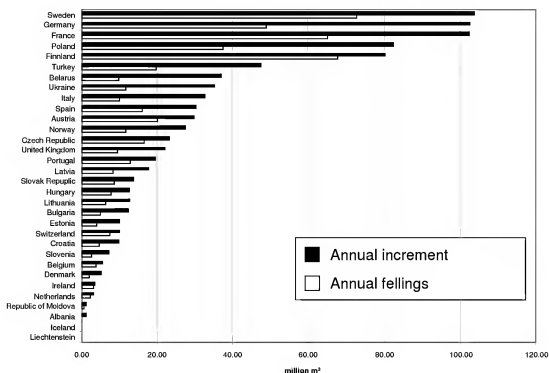


Fig. 1 Annual fellings and annual increment in European countries without Russian Federation (MCPFE & UNECE/FAO 2003)

However, two desk studies of the pulp and paper industry provide a different assessment. The studies predict that the introduction of the “close to nature forest management” in Europe will lead – between 1990-2090 and 2005-2060 – to an increase of the area of deciduous trees. The shortfall in supply for coniferous wood will therefore rise and will be significantly noticeable (see Fig. 2). By 2090, forest management might be on the brink of becoming unsustainable in some regions in Europe (Nabuurs et al. 2002, 2003).

In a study from the UNECE/FAO (2003), projections for demand, supply and trade in Europe were made for the period 2000 - 2020. In comparison with the older European Timber Trend Study V (UNECE/FAO 1996), the scope was extended by 17 more countries (CEEC= Central and Eastern European Countries and CIS= Commonwealth of Independent States). The study confirms the increasing wood consumption in Europe, but predicts decreasing growth rates. In general, consumption of forest products is growing slower than the economy as a whole. The trade between countries will increase and market structures will change. In 2020, more than half of the sawnwood produced in the region (EU/EFTA, CEEC, CIS) will be manufactured solely in the CEEC and CIS countries. The EU (European Union) and the countries of the European Free

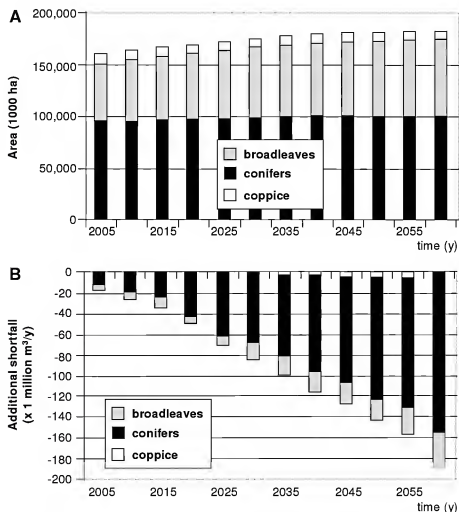


Fig. 2 A. Development of the area by main species group and B. projected additional virtual shortfall in supply for all wood commodities under "new management trends" in Europe excluding Russia (Nabuurs et al. 2003)

Trade Association (EFTA) will remain dominant in the paper production with a proportion of about 80%.

Wood supply in Germany

Germany is one of the leading nations in forestry and forest industries in Europe concerning wood production and wood utilisation.

The total area of forests in Germany was about 11.1 million hectares in 2002. The growing stock amounted to nearly 3.4 billions m³ and the harvesting volume to 54.5 million m³ per year (BMVEL 2004a, UNECE/FAO 2000). With these data, Germany is ranking on 4th, 1st, and 1st place in Europe. With an average

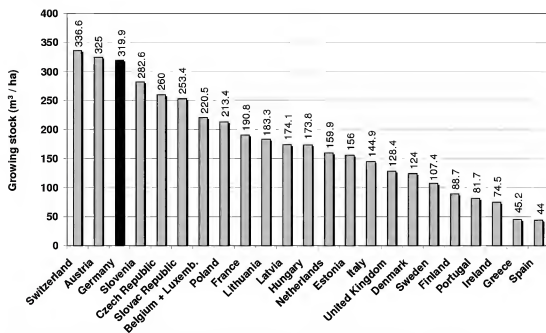


Fig. 3 Growing stock per hectare in European countries (Polley et al. 2004)

growing stock per hectare of about 320 m³, Germany is on 3rd place behind Switzerland and Austria in comparison with the other European countries (MCPFE & UNECE/FAO 2003; see Fig. 3).

Within Germany, the growing stock reaches from 237 m³ per hectare in Saxony-Anhalt up to 403 m³ per hectare in Bavaria, according to the results of the second National Forest Inventory in 2002 (BMVEL 2004b). Comparing the former federal states of Germany before unification, Bavaria, North Rhine-Westphalia, and Lower Saxony were the ones with the highest increase of growing stock between 1987 and 2002.

Throughout Germany, the coniferous stands dominate with a proportion of 63.8% of the growing stock. The Norway spruce (*Picea abies*) has the highest growing stock as well as the largest growing area, followed - by a larger distance - by pine (*Pinus sylvestris*) and beech (*Fagus sylvatica*) (BMVEL 2004b; see Fig. 4).

In Germany, a considerable part of the growing stock had target diameter dimensions in 2002. 600 million m³ of large sized timber with a diameter at breast height (dbh) above 50 cm were identified, which corresponded to around 18% of the growing stock. The growing stock of large sized timber above 50 cm dbh increased by 72% since 1987. Fir (*Abies alba*), beech (*Fagus sylvatica*) and oak (*Quercus robur*, *Quercus petraea*) have the highest proportion of large sized timber (see Fig. 5). Pine has the lowest proportion, which has to be evaluated in connection with the growing sites and the age class distributions.

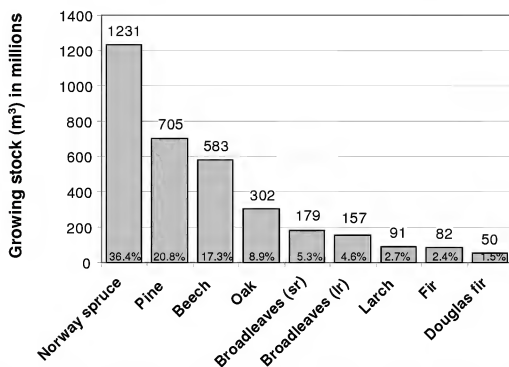


Fig. 4 Growing stock by tree species (BMVEL 2004b); (sr: short rotation; lr: long rotation)

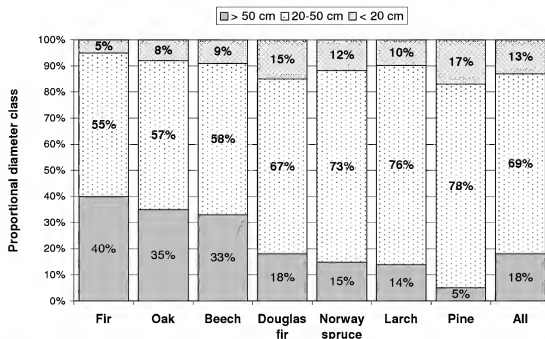


Fig. 5 Distribution of diameter classes in total growing stock by tree species (Polley et al. 2004)

In Germany, the wood balance is negative because the domestic production of only 54.5 million m³ in the year 2004 faced a total consumption of 103.6 million m³ (see Fig. 6). This shortage has been covered by both, an increasing domestic supply of scrap-wood and waste paper as well as higher imports. Despite of increasing exports by volume as well as by values, Germany still continues to act as a net importer of timber and timber products. The globalisation process more and more affects the German forest and wood industry (Thoroe & Ollmann 2001, Merker 2003, Dieter 2005).

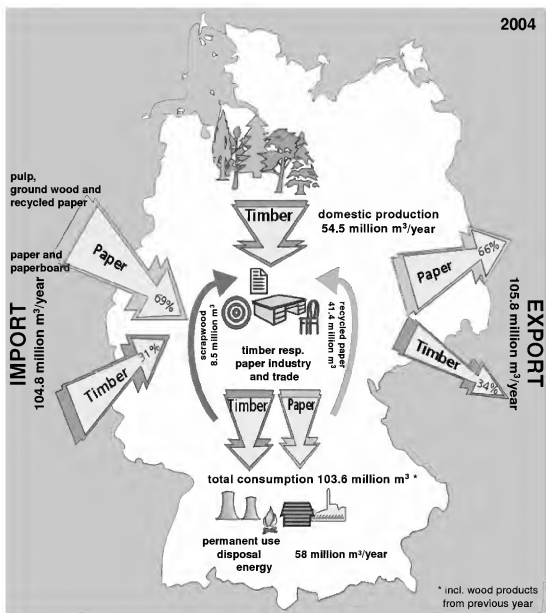


Fig. 6 Timber balance in Germany 2004 (Dieter 2005)

The German sawnwood, particle board and paper industry ranks at the first place among the European competitors with its production volumes of 19.5 million m^3 , 10.3 million m^3 and 20.4 million m^3 , respectively (ZMP 2005). Particularly the vicinity to the envisaged markets, the amounts of mobilisable wood resources, fitting traffic links and subsidies strengthened the production position of East Germany and enabled to build up large production capacities (Merker 2003).

According to provisional data for 2004, Germany has been a net exporter for the first time regarding coniferous and deciduous sawnwood (ZMP 2005). Due to the export, the coniferous sawnwood sector has developed more and more into the most important production field. By the transition from the frame saw to the chipper-canter technology, globally oriented production capacities have been set up which accelerated the concentration processes in the local wood industry. Instead of producing several special types of construction sawnwood, more and more standardised sawnwood is manufactured, internationally traded and sold. At the same time, the roundwood demand is increasing with a changing structure of assortments (Starke 2003). The number of sawmills decreases at a high rate, whereas the production capacities of the remaining and newly built sawmills are going up, so that - nevertheless - more wood arrives at the market.

The statistics of the wood market indicate that the supply of coniferous round timber increased from 15 million m^3 in 1985 to 28.7 million m^3 in 2004 whereas the deciduous roundwood supply remained between 3 and 5 million m^3 (see Fig. 7). The coniferous industrial wood supply increased from 8 million m^3 to

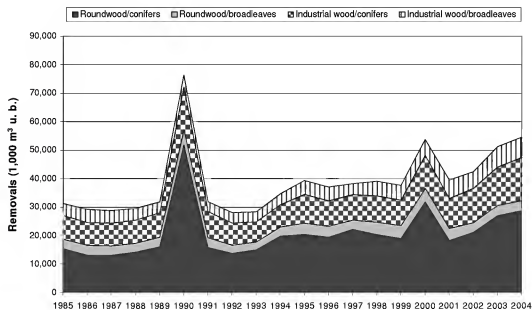


Fig. 7 Harvest volume of roundwood and industrial wood in Germany in the years 1985 – 2004 (ZMP 2005); u. b. = under bark

15 million m³ and the deciduous wood from 4 to 7 million m³ (ZMP 2005). Norway spruce is - as ever - the most important tree species of the German forest economy, accounting for 50 to 70% of the roundwood harvesting volume and sales. In the last years, the market for beech has been subject to a strong fashion trend. Between 1999 and 2002, supply and prices came up to record heights, stimulated by the China business. In the meantime, numerous small- and middle-sized beech sawmillers went bankrupt by weakened demand. In contrast to this, coniferous industrial wood achieved a significantly increase in demand by newly built paper and wood-based panel industries as well as by reinforced energetic use.

Regarding the domestic utilisation of deciduous and coniferous wood, in the past 20 years only coniferous sawnwood achieved a significant increase from 7.9 to 18.3 million m³, whereas the values from deciduous sawnwood stayed almost unchanged between 1.5 (1985) and 1.1 million m³ in 2004 (ZMP 2005). In relation to the German wood market, the shares of veneer and plywood consumption are infinitely small. In this market, there are no major changes expected, because the differences in the consumption of deciduous and coniferous wood are primarily not explained by the supply but by the different wood properties. Chances for deciduous wood products arise rather from the revival of the exports to overseas.

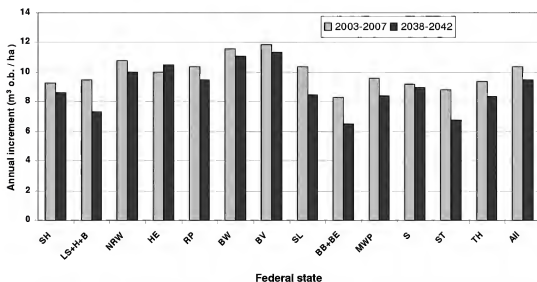


Fig. 8 Results of increment prognosis with WEHAM on basis of the National Forest Inventory II (BMVEL 2004b); B: Bremen, BE: Berlin, BV: Bavaria, BW: Baden-Wuerttemberg, H: Hamburg, HE: Hesse, NRW: North Rhine-Westphalia, TH: Thuringia, RP: Rhineland-Palatinate, SH: Schleswig-Holstein, MWP: Mecklenburg-Western-Pomerania, LS: Lower Saxony, SL: Saarland, S: Saxony, BB: Brandenburg, ST: Saxony-Anhalt; o.b. = over bark

The model WEHAM (“Waldentwicklungs- und Holzaufkommensmodellierung”) simulates the development of the German forests and the wood supply based on the data from the second National Forest Inventory. It predicts a high potential supply of roundwood with an average of 78 million m³ (under bark) per year and an increase of the growing stock up to 326 m³ per hectare, both between 2003 and 2042 (BMVEL 2004b). Furthermore, a decrease of the annual increment from 10.36 to 9.46 m³ per year and hectare is predicted as an average for all federal states (see Fig. 8). This alteration is mainly due to the shifting of the age class distributions towards older forest stands. In consequence of this, the proportion of larger sized assortments is going up.

In the future, the main species groups spruce/douglas fir and beech will have the highest potential of annual felling (see Fig. 9). The douglas fir will gain importance within the main species group spruce/douglas fir. The potential of annual felling of the douglas fir will already exceed in the period 2013 to 2017 that of spruce (BMVEL 2004b).

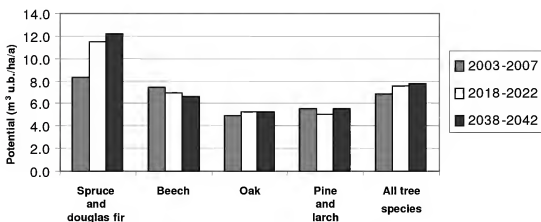


Fig. 9 Potential of annual felling for main species in Germany predicted with the aid of the model WEHAM on basis of the National Forest Inventory II (BMVEL 2004b); u.b. = under bark

Private ownership is the predominant type of forest ownership in Germany. 43.6% of the total forest area are private forests, 29.6% federal forests and 19.5% corporation forests. The highest potential of annual fellings are found in private owned forests and in the corporation forests (see Fig. 10). The mobilisation of the roundwood potential in the predominant privately owned forests will be of decisive importance for the future wood supply in Germany.

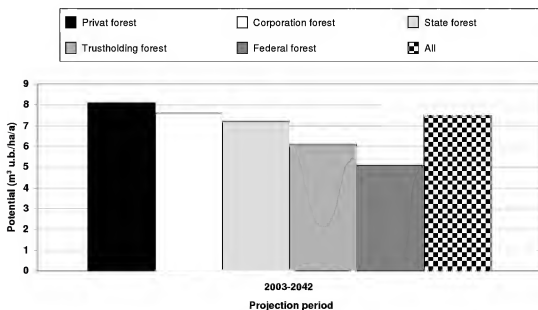


Fig. 10 Potential of annual felling for different ownership types in Germany predicted with the aid of the model WEHAM on basis of the National Forest Inventory II (BMVEL 2004b); u.b. = under bark

Wood supply in Lower Saxony

With a total forest area of 1.16 million hectares, Lower Saxony ranks on the third place behind Baden-Wuerttemberg and Bavaria, in comparison with the other German federal states. However, with a share of forests of the total land area of 24.3%, Lower Saxony is the third from behind (Niedersächsische Landesforsten 2004).

The average growing stock amounts to about 260 m³ per hectare and the average increment to 10.6 m³ per hectare and year (Niedersächsische Landesforsten 2004; BMVEL 2004b). About 4.5 million m³ (under bark) are harvested annually equivalent to 4.3 m³ (under bark) per hectare. The annual harvesting volume ranges far below the annual increment in Lower Saxony. This is not only due to the age class distribution but also because of problems with the mobilisation of wood, as a consequence of structural problems of the small sized private forests. 59% of the total land area of Lower Saxony are private or corporation forests. 80% of the private forests are managed by enterprises with an area below 200 hectares, almost half of the enterprises have an area even below 20 hectares. About 18% have forest sizes between 1 and 5 hectares (Niedersächsische Landesforsten 2004). 57% of the total growing stock of the private forests are located in small sized private forest enterprises with an area below 50 hectares (see

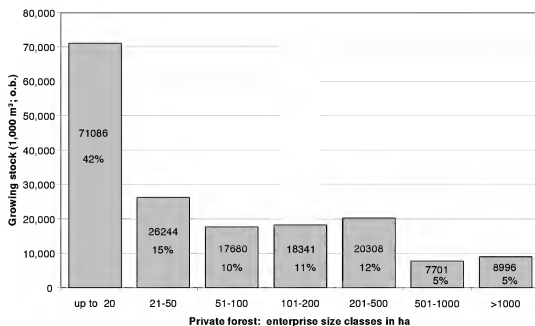


Fig. 11 Growing stock in private forests by enterprise size classes (Niedersächsische Landesforsten 2004); o.b. = over bark

Fig. 11). Mobilising this roundwood potential is one of the most important tasks of the future.

Under the assumption of a continuous species mix, the prognosis of wood supply predicted an increase of the growing stock up to 291 m³ per hectare for the period from 2002 to 2042, according to WEHAM for Lower Saxony, Hamburg and Bremen. The annual increment will decrease from 9.5 to 7.3 m³ per hectare (BMVEL 2004b) from the first calculated period (2003-2007) to the last (2038-2042), mainly because of the increasing proportion of older stands.

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3. Development and Trends of Wood Production in the Tropics

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Introduction

The available data on wood production in the tropics are in part contradictory, which can be explained by the lack of relatively reliable statistics in many countries and different definitions of the general terms “tropics” and “wood”. The most recent and most reliable data in this context are those compiled by the Food and Agriculture Organization of the United Nations (FAO). Tropical timber accounts only for a small portion of world trade and world timber production; however, the economies of many developing countries in the tropics rely heavily on it (FAO 1995a). For example, Malaysia exported 61% of the produced sawnwood whereas export of plywood in Indonesia accounted for 78% of the production in 2003 (FAOSTAT data 2005). Other tropical countries having high export share include

Congo, Côte d'Ivoire, Gabon, Ghana, Liberia, Papua New Guinea, etc. (FAO 1995a). Wood industries in many non-tropical countries import large amounts of tropical timber for domestic use or re-exporting. China, Taiwan Province of China (P.O.C.), and Republic of Korea are the major importers of tropical industrial roundwood, sawnwood, veneer and plywood as in 2003 (ITTO 2005). The importance of production and trade of tropical timber is reflected by the intergovernmental organisation ITTO – the International Tropical Timber Organization. Concerned with increasing deforestation and recognising the importance of tropical forests in providing different goods and services, ITTO develops internationally agreed policy documents to promote sustainable forest management and conservation, and to analyse trade and production of tropical timber (ITTO 2005).

Tropical forests serve as a primary source of energy to many livelihoods in tropical nations where other energy sources are not available or at higher cost. In these countries, fuelwood is collected for household energy; nutritional problems will arise if there is inadequate availability of fuelwood. Wood is also used for charcoal production as this is another important source of energy. In 1995, it was estimated that 3,000 million people depended primarily on fuelwood and charcoal for energy (FAO 1995a). In 2005, woodfuel, consisting of fuelwood and charcoal, accounts for 53% of total roundwood production in the world (FAO 2005a).

In this chapter, production time series are provided for many primary forest products: industrial roundwood, sawnwood, veneer sheets and plywood. A trend in woodfuel production is included in the analysis as its importance should not be overlooked. Two major events have significantly affected the trade of tropical timber: the Asian economic crisis, and logging and trade ban. Their effects are summarised briefly within the text. As the role of plantation is increasingly important (see also Chapter 4 of this book), two interconnected issues are discussed to provide some insights to related problems. Finally, a section on future trends in tropical forest production and utility conclude this chapter.

Definitions

Tropical countries

A definition of the tropics is essential to construct production and utility statistics; however, the definition is unequivocal and inconsistent between publications. When assessing plantation in the tropical region, FAO (2003) classified tropical countries as countries having more than 50% of their land area between Tropic of Cancer and Tropic of Capricorn. Nonetheless, this analysis did not include all countries within this zone. Table 1 indicates the tropical countries included in this chapter following the above definition in FAO (2003) plus adjustment from Forest Resource Assessment 1990 – Tropical Countries in 1990 (FAO 1993). In this chapter, a total of 91 countries is analysed with 40, 15 and 26 countries from the Africa, the Asia-Pacific, and the Latin America-Caribbean region, respectively.

Table 1 List of tropical countries for each region included for data analyses in this chapter

Africa	Nigeria	Latin America and Caribbean
	Rwanda	
Angola	Senegal	Bahamas
Benin	Sierra Leone	Belize
Botswana	Somalia	Bolivia
Burkina Faso	Sudan	Brazil
Burundi	United Republic of Tanzania	Colombia
Cameroon	Togo	Costa Rica
Cape Verde	Uganda	Cuba
Central African Republic	Zambia	Dominican Republic
Chad	Zimbabwe	Ecuador
Democratic Republic of Congo		El Salvador
Republic of the Congo		French Guiana
Côte d'Ivoire	Asia and Pacific	Guadeloupe
		Guatemala
Djibouti	Bangladesh	Guyana
Equatorial Guinea	Bhutan	Haiti
Eritrea	Brunei Darussalam	Honduras
Ethiopia	Cambodia	Jamaica
Gabon	India	Martinique
Gambia	Indonesia	Mexico
Ghana	Laos	Nicaragua
Guinea	Malaysia	Panama
Guinea-Bissau	Myanmar	Paraguay
Kenya	Nepal	Peru
Liberia	Pakistan	Suriname
Madagascar	Philippines	Trinidad and Tobago
Malawi	Sri Lanka	Bolivar Rep. of Venezuela
Mali	Singapore	
Mauritania	Thailand	
Mozambique	Viet Nam	
Niger		

Wood

Wood includes **roundwood**, **woodfuel**, **industrial roundwood**, **sawnwood**, **veneer sheets** and **plywood**, which can be defined as follows (FAO 2005b). Roundwood is the quantity of wood obtained from removal, harvesting and felling from forest and trees outside forest. It includes roundwood generally classified as woodfuel (fuelwood and charcoal) and industrial roundwood (sawlogs and veneer logs, pulpwood, round and split, and other industrial roundwood). Woodfuel is the quantity of roundwood that is used for fuel consumption such as

cooking, heating or power production. Industrial roundwood is defined as the quantity of roundwood, which is used for industrial production of other goods and services except as a source of energy. It includes several products: sawlogs and veneer logs (production of sawnwood or railway sleepers and veneer sheets, respectively), pulpwood, round and split (pulp, particleboard or fibreboard), and other industrial roundwood (tanning, distillation, poles, etc.). Sawnwood is the quantity of wood that has been produced from domestic and imported roundwood either by sawing lengthways or by a profile-chipping process. Veneer sheets are thin sheets of wood of uniform thickness peeled, sliced or sawn. Finally, plywood is a panel consisting of an assembly of veneer sheets bonded together with the direction of the grain in alternate piles generally at right angle (FAO 2005b; see also Chapter of 15 of this book).

Production Statistics

All production statistics are quoted directly or derived from FAOSTAT data available online (FAOSTAT data 2005). The time series are constructed from 1961 to 2004. FAOSTAT data are collected annually from countries reports and are reported in the FAO Yearbook of Forest Products (FAO 2005b). Further detailed definition and information can be obtained from the publication.

Roundwood

Production of roundwood in both Africa and the Latin America-Caribbean region increased steadily from 1961 to 2004. In the Asia-Pacific region, however, the production increased slower compared to the other regions and decreased after 1998 (Fig. 1). Roundwood production in the Asia-Pacific region was the highest among the three regions, followed by Africa and the Latin America-Caribbean region. Production of industrial roundwood differed between the three areas over 44 years, but woodfuel consistently constituted a large portion of this production for all regions (Fig. 2).

Production of roundwood in Africa increased from 249,126,000 m³ in 1961 to 556,934,000 m³ in 2004. This represents a 124% increase in total and an average annual production rate of 6,995,000 m³/year. Production of industrial roundwood increased from 19,392,000 m³ to 48,399,000 m³ by the end of 2004 (a 150% increase) but maintained a relatively constant share to total roundwood production (in average 9.15%). Among the three different areas in the world, woodfuel had the largest share of total roundwood production in Africa – this amounts to an average of 90.85% over 44 years (Fig. 2A) and reflects the importance of wood as a source of energy in Africa. A study by the International Energy Agency (2003) revealed that the Africa region had the highest contribution of woodfuel (about 25%) to total primary energy supply in 2001 among different regions in the world.

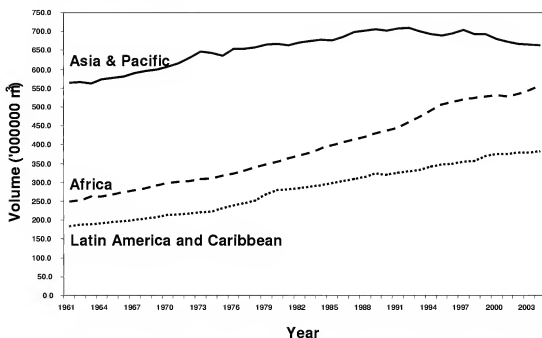


Fig. 1 Roundwood production in three tropical regions from 1961 to 2004. Curves with continuous, dashed and dotted lines depict tropical Asia & Pacific, Africa, and the Latin America & Caribbean region, respectively

The increase in roundwood production in Latin America-Caribbean region roughly mirrors the production in Africa (Fig. 1). The production went up from 184,284,000 m³ in 1961 to 382,458,000 m³ in 2004. This amounts to an increase of 108%, with an average annual production rate of 4,504,000 m³. However, the share of woodfuel and industrial roundwood to total roundwood production was different to other regions (Fig. 2C). The share of industrial roundwood increased from 15.57% in 1961 to 26.30% in 2004 while the share of woodfuel dropped by 17.5% at the end of 2004. Nonetheless, woodfuel production increased steadily over the years but only with a total value of 65%. As for industrial roundwood, there seemed to have been a boom between 1974 and 1981 as the production increased sharply during that period (Fig. 2C).

Unlike roundwood production in the other two regions, the production in the Asia-Pacific region fluctuated over the years. From 1961 to 1997, the roundwood production increased from 564,811,000 m³ to 703,904,000 m³ (a total increase of 24.63%), with an average production annual rate of 3,759,000 m³. However, the production decreased onwards to 664,299,000 m³ by the end of 2004 (a decrease of 5.63%). Fig. 2B shows that the woodfuel production was relatively constant over 44 years with only a slight increase from 527,741,000 m³ to 570,101,000 m³ (a total increase of 8.03%) but the share to total roundwood production varied over the years. This was caused by irregular industrial roundwood production. On the

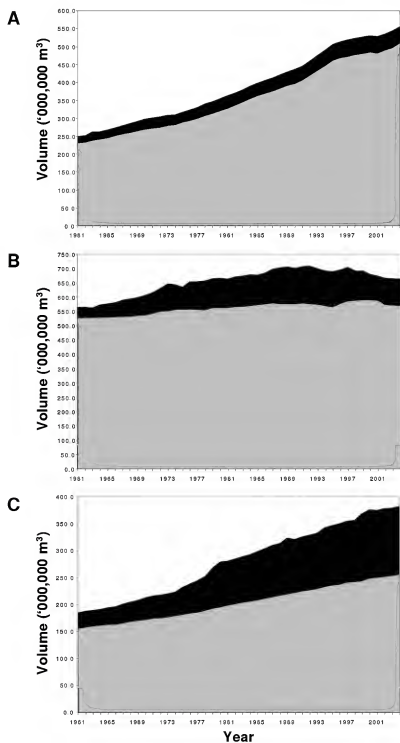


Fig. 2 Woodfuel and industrial roundwood production in three tropical regions from 1961 to 2004: (A) Africa, (B) Asia-Pacific and (C) Latin America-Caribbean region. Black and grey shaded areas depict industrial roundwood and woodfuel production, respectively

average, the share of industrial roundwood over the years was 14.01% with a minimum of 6.17% and a maximum of 18.79%. The share of industrial roundwood grew steadily from 1961 to early 1980's but decreased and fluctuated afterwards (Fig. 2B). Industrial roundwood production generally increased from 37,070,000 m³ in 1961 and reached its peak of 133,186,000 m³ (a 259.3% total increase and an average production annual rate of 3,004,000 m³) in 1992. Subsequently, it continuously decreased to a value of 94,198,000 m³ at the end of 2004. The production at 2004 was equivalent to the production during the mid 1970's. It is speculated that the decrease is the effect from the economic crisis experienced by various Asian countries.

Sawnwood

Sawnwood production is different among the three tropical regions. In Africa, the production rised from 2,028,000 m³ in 1961 to 5,935,000 m³ in 2004 - however, the annual rate of increase (in average 88,784 m³/year) was the lowest among the

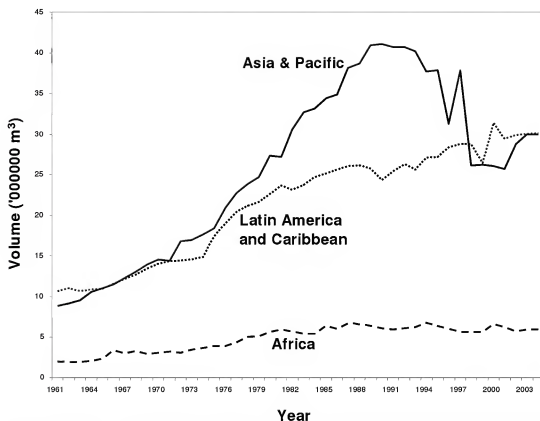


Fig. 3 Sawnwood production in three tropical regions from 1961 to 2004. Curves with continuous, dashed and dotted lines depict tropical Asia & Pacific, Africa, and the Latin American & Caribbean region, respectively

three regions. With slight variation, the production after the mid 1980's reached a plateau of an averaged 5,500,000 m³ (Fig. 3).

From 1961 to 2004, the Latin America-Caribbean region saw a significant and steady growth of sawnwood production from 10,676,000 m³ to 30,160,000 m³, with an average annual production rate of 443,000 m³. In this region, there was a sudden boom of production after 1975, that caught even up with that of the Asia-Pacific region in 2004 (Fig. 3).

The Asia-Pacific region was the dominant player in sawnwood production until 1998. Thereafter, the production fell below that of the Latin America-Caribbean region (Fig. 3). This effect is likely also caused by the Asian economic crisis. In the Asia-Pacific region, there was a boom of production after 1971 that reached its peak in 1990. The production increased from 8,887,000 m³ in 1961 to 41,122,000 m³ in 1990 with an average annual production rate of 1,074,000 m³. The trend of decrease fluctuated highly after 1990: there was a sharp decrease in 1996 but the production in 1997 bounced back to the level of 1995 (Fig. 3). The production in 1998 decreased sharply below that of the Latin America-Caribbean region. The recovery after 1998 brought the production to the level of 29,981,000 m³ in 2004 which is however only 72.9% of the value in 1990 (Fig. 3).

Veneer Sheets

Veneer sheet production in all three tropical regions was dynamic throughout several decades.

Before 1990, the Africa region had a higher production than the Latin America-Caribbean region and the production increased steadily. After 1990, the production was overtaken by the latter region, although there was a remarkable boom in the African production after 1998 (Fig. 4). The African production grew from 75,000 m³ in 1961 to 772,000 m³ in 2004, with an average annual production rate of 16,000 m³.

The production in the Latin America-Caribbean region increased steadily from 1961 to 1996. There was a sudden peak of production (1,750,000 m³) in 1997, but it dropped in the next year to again increase steadily after that (Fig. 4). The production rose from 37,000 m³ in 1961 to 1,323,000 m³ in 2004, a value of roughly twice the amount in Africa. Excluding the production in 1997, the average annual rate of production was 30,000 m³.

The Asia-Pacific region always dominated the market of veneer production from 1961 to 1993 after which the production decreased sharply (Fig. 4). The production increased from 61,000 m³ in 1961 and reached a maximum of 2,387,000 m³ in 1993, with an average annual production rate for this period of 71,000 m³. The production between 1998 and 2004 fluctuated a lot and was in a range similar to that of the Latin America-Caribbean region (Fig. 4). The production in 2004 was 1,522,000 m³ and thus only slightly higher than that of the Latin

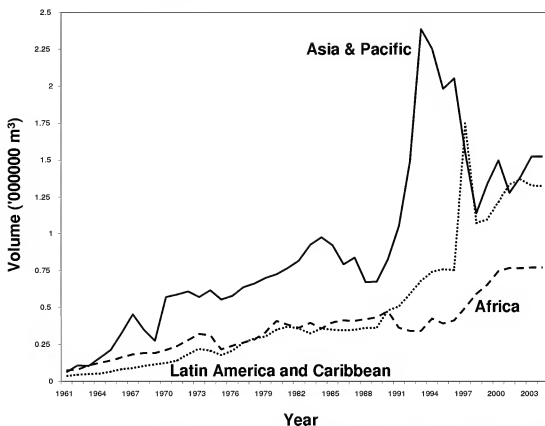


Fig. 4 Veneer sheets production in three tropical regions from 1961 to 2004. Curves with continuous, dashed and dotted lines depict tropical Asia & Pacific, Africa, and the Latin America & Caribbean region, respectively

America-Caribbean region. The production of the Asia-Pacific region in 2004 was just half of the production presented in 1993.

Plywood

The Asia-Pacific region is the sole player of importance in plywood production among the three different tropical regions while the Africa region has a neglectable role (Fig. 5).

In Africa, the production increased slowly from 102,000 m³ in 1961 to 491,000 m³ in 2004 with an average annual production rate of only 8,000 m³. The production in 2004 was only 3.57% of the production in the Asia-Pacific region of the same year.

The production in the Latin America-Caribbean region has also not been very high but seen a steady climb towards 2004 with a sharp decrease in 1997 and a recovery after that (Fig. 5). The production increased from 315,000 m³ in 1961 to

3,287,000 m³ in 2004 with an average annual production rate of 67,000 m³. In 2004, the production was about a quarter of that in the Asia-Pacific region (23.94%).

In contrast, the production in the Asia-Pacific region increased sharply from 1961 to reach its peak in 1997 followed by a large drop and a recovery (Fig. 5). The production from 1961 to 1997 climbed from 224,000 m³ to 15,183,000 m³ with an average annual production rate of 404,000 m³. The decrease in production from 1997 to 1998 was 17.15%, with 12,472,000 m³ total production at the end of 1998. The slow recovery brought the production back to 13,734,000 m³ at the end of 2004 which is 90.46% of the peak production from 1997. Even with the Asian economic crisis, Asia and the Pacific region still continued to dominate the market of tropical plywood (Fig. 5).

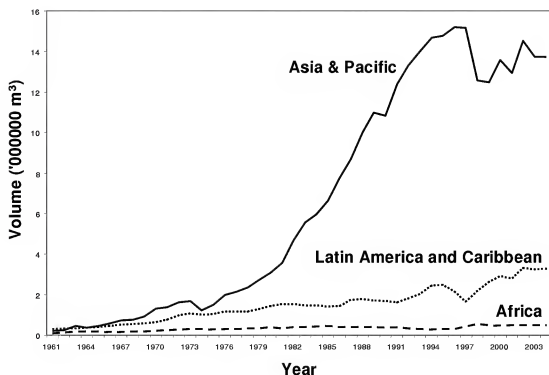


Fig. 5 Plywood production in three tropical regions from 1961 to 2004. Curves with continuous, dashed and dotted lines depict tropical Asia & Pacific, Africa, and the Latin America & Caribbean region, respectively

Effects of major events on trade

The Asian Economic Crisis

The Asian economic crisis began with significant depreciation of a number of key Asian currencies in mid-1997. Until about September 1998, the effect of the economic crisis was largely confined to the Asia-Pacific region, but there were signs that the effect started to spread to other regions and countries that relied heavily on tropical timber export from the Asia-Pacific region. One of the main effects was reduced demand for all forest products, affecting most significantly China, Japan, the Republic of Korea, and Thailand. By that time, Asia accounted for about 80 percent of tropical wood exports and more than 70 percent of tropical wood imports by value; thus, the market and trade of tropical forest products were severely disrupted (FAO 1999).

Another effect was the increasing competitiveness of affected exporting countries through exchange rate depreciation, but in the face of reduced demand. Coupling with price drops, other tropical regions were affected by the recession that caused a shift in market shares. During the economic crisis, exporters in the Asia-Pacific region tried to maintain sales by lowering the prices of many forest products. This increased competitiveness not only among affected Asian countries but also African and South American countries because importers preferred lower priced Asian products. For example, South American exporters were affected by cheaper Asian plywood. Other effects included reduced earning in the forestry sector resulting in closure of mills, reduced harvests and workforce lay-offs and changes of Indonesian forest policies resulting from the Indonesian government's efforts to meet International Monetary Fund loan requirements (FAO 1999).

Logging and Trade Ban

Inclusion of mahogany species (*Swietenia* spp.) in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) in 2003 affects the trade in mahogany (ITTO 2005). With the ban of logging, transportation, processing and exporting of Brazilian mahogany species (*Swietenia* spp.), other species such as African mahogany (*Khaya* spp.), also known as khaya, received larger attention, especially with the USA continuously absorbing most of the khaya made available in the market. The price for African mahogany continued to rise till the end of 2004 (ITTO 2005). Although mahogany can still be harvested from approved Sustainable Forest Management in Brazil since mid-2003, the international market has been replaced by Peruvian mahogany (ITTO 2005), which in principle includes the same species of the genus *Swietenia*. In summary, the logging and trade ban on specific species causes the market to look for new substitutes and it changes the current prices.

Indonesia imposed an export ban on logs since 2002 and further implemented a ban on sawnwood since late 2004. In addition, Indonesia tightened controls to

regulate illegal trade (ITTO 2005). This caused that the Indonesia export of tropical log shrunk to 100,000 m³ in 2003 which is an 85% drop from the 2002 level (ITTO 2005). With the ban on sawnwood, another drop in export occurred in late 2004. The effect of such bans strengthens however the export of other countries and causes shifts in market shares. Export of sawnwood by Malaysia for example has risen at the end of 2004 (ITTO 2005).

Tropical plantation

Plantation plays a significant role for wood production in tropical countries because of its several important characteristics: higher yield per unit area, very short rotations (7-30 years) compared to natural forests (30-150 years), better accessibility, etc. (FAO 1995b). Resulting from these factors is the increasing share of plantation in providing roundwood to the industries: 60% and 50% of roundwood production in Brazil and Zimbabwe, respectively, comes from plantation (FAO 1995b). Plantation in a narrow sense refers to industrial plantation established to provide goods such as sawlogs and veneer logs. As community and non-forestry plantation are gaining momentum over several decades, trees planted outside forests either for domestic or industrial purposes are included in the definition of plantation (for further reading and discussion see Chapter 4 of this book). Plantation discussed within this text takes the latter approach. Two issues relating to plantation that warrant attention are net plantation area and growth and yield.

Net Plantation Area

Although many countries have reports on plantation area, it is essential to distinguish successful plantation area from total planted area. Wiersum (1984) identified several main factors leading to failure of plantations: lack of planning, inadequate supervision, insufficient or untimely allocation of funds, etc.. Where a plantation project is supported by agencies, sometimes more attention has been paid to achieving the required target in terms of area rather than planting quality. In addition, failure to protect and aftercare after planting in the presence of inadequate funding contributes to failure of plantation as well (FAO 1995b). The net plantation area is defined as the successful planted area. The success rate in Africa, the Asia-Pacific region, and the Latin America-Caribbean region is estimated to be 70%, 61% and 84%, respectively (FAO 2003).

However, these regional estimates hide country variations. For example, Brazil has 87% of success rate whereas it is only 57% for Colombia. In the Asia-Pacific region, the Philippines have a success rate of only 26% whereas Sri Lanka has a higher success rate of 70% (FAO 2003). Nonetheless, it is speculated that the success rate for industrial plantation will be higher than for community established and non-forestry plantation. Users should practice extreme caution when applying these estimates as the quality and sufficiency of data used to derive these estimates are doubtful (FAO 1995b).

Growth and Yield

Reports on the growth and yield of plantations have mostly been over-optimistic. The reason is that existing yield tables are prepared for fully stocked stands, good site quality and careful management. In addition, future yield estimates are often derived by extrapolating the performance of young plantations. Another motivation for project proposals to overestimate plantation yields is to attract financial support. However, this does not necessarily imply that a plantation cannot reach its full potential. This can be achieved through careful planning such as choice of site condition, species, provenance, spacing, rotation age, thinning, tree breeding, and aftercare.

Successful large scale plantations such as *Eucalyptus grandis* in Aracruz, Brazil, can have mean annual wood increments of up to 70 m³/ha. However, a poor management can lead to yields as low as 1 m³/ha/year. Comparisons between optimum, actual and potential yields are given in the following for teak and pine. The optimum yield is extrapolated from permanent sample plot studies. The actual yield is given as the yield obtained from established large scale plantation whilst the potential yield is given as the yield from natural forest (FAO 1995b).

Teak (*Tectona grandis*) is one of the internationally well-known and valuable timber species (see also Chapter 4 of this book). Cultivation of teak goes back to 1868, when the German botanist Dietrich Brandis established plantations in Birma using the so-called Taungya system. Plantations of teak in Java, Indonesia have also a long history and go back to 1880. Teak is still grown as the prevalent plantation species of valuable timber. The optimum yield of teak varies with site quality and rotation age. Perum Perhutani, an Indonesian forest enterprise, is reducing the rotation length in teak plantation from formerly 80 to nowadays 40 years. For the poorest site, the lowest optimum mean annual increment (MAI) is 6.2 m³/ha/year at a 40-year rotation while a higher MAI can be achieved by increasing rotation length. On the best site, the MAI of teak can reach the highest value of 18.7 m³/ha/year at a 40-year rotation (FAO 1995b). In comparison, the potential yield from natural forest in Java is estimated to be 12.9 m³/ha/year. In India, the optimum yield from plantations ranges from 2.0 m³/ha/year to 9.4 m³/ha/year, from poor to best site quality. The actual yield from a specific felling site in the Konni division at a 70-year rotation is estimated to be 2.5 m³/ha/year. In natural forest of Kerala State, India, the MAI for teak is estimated to be 9.0 m³/ha/year (FAO 1995b). Teak is planted mainly for production of high valued timber rather than for maximum growth. However, it is possible for teak in plantation to obtain as high yields as in natural forest. It is speculated that the poor yield from plantation in Indonesia and India is due to a poor initial stocking. Factors such as competition for landuse, i.e. agriculture, illegal felling, grazing and fire are speculated to contribute to the poor yield as well (FAO 1995b).

The genus *Pinus* can be considered as one of the more widely planted genus of the conifers and tropical pine species play an important role in plantation forestry (see also Chapter 4 of this book). There are at least seven species of pines exhaustively planted in the tropics for production of industrial wood – mainly for pulp production (*Pinus caribaea*, *Pinus elliotti*, *Pinus oocarpa*, *Pinus patula*, and *Pinus radiata* originated all from Central and North America; *Pinus kesiya* and *Pinus merkusii* from Asia). Madagascar is one of the countries in the Africa region with a sizeable pine plantation area, a large proportion of which was established under World Bank financed projects. However, optimistic prediction by experts, haste and incorrect choice of species and non-application of proper silviculture management are identified as the factors leading to a poor yield. In a detailed field inventory during 1982, the mean annual increment for the pine plantation was estimated to be 5.68 m³/ha, whereas the potential yield from a natural forest is estimated to be 10 m³/ha/year (FAO 1995b). This example shows that without proper management, the yield of a plantation may be lower than the yield in natural forests. In contrast in Brazil, pine is the second largest species group planted and most of the plantations are owned and managed by the private sector. On a 20-year rotation, the MAI of the actual yield in plantation is estimated to be 15 m³/ha/year whereas the potential yield of a natural forest is estimated to be 11 m³/ha/year (FAO 1995b).

Future outlook

In near future, the role of plantation will gain more importance and the area under plantation is expected to increase. For one reason, there is a change in attitude towards natural forest in wood production due to concern in deforestation and degradation of forest land. For example, the Philippines has banned all logging in “old growth and virgin forests” and placed such forests under the National Integrated Protected Area System (FAO 1999). Suriname sets aside 1.5 million hectare of natural forest (equivalent to one-tenth of the country land area) as a Wilderness Nature Reserve in 1998 (FAO 1999). Another factor that causes the removal of natural forest from wood production is the change in ownerships. For example, in Amazon regions, large areas of natural forests are relocated for the use of native communities. It is expected that relocation of land ownerships to smaller owners prevents large scale wood clearing. With the amendment of the Kyoto Protocol in 2005, it is expected that plantations will act as carbon sink in many carbon credit projects (see Chapter 5 in this book). Various factors will cause the gradual shift from forests undisturbed by humans to semi-natural forests, plantations and trees outside forest. However, it should be noted that plantations are not a panacea for deforestation and nor can they replace all functions of natural forests (further reading in Chapter 4 of this book). Therefore, there should be careful management of both natural and plantation forests.

It is very likely that the roundwood production in tropical countries will increase in near future as demand does not wane. A significant contributor to such increase is woodfuel consumption. FAO (2005a) expected the global woodfuel production to increase moderately in near future as dependency of woodfuel in developing countries is still high. Most households in these countries resort to using wood as main energy source because their choices of energy supply are restricted by income and availability of other types of energy sources. Many studies have found a correlation between income and woodfuel consumption. In very poor rural households in Brazil, India, Pakistan and Sri Lanka, the woodfuel consumption increases as income increases (Leach et al. 1986). However, other studies have shown the contrary (Sathaye and Tyler 1991, Leach 1988, Broadhead et al. 2001). Thus, it is not always that low-income household will switch to different types of energy sources as income increases. The change will depend on prices, availability and reliability of supply and cost of changing equipment. Consequently, the change from woodfuel to other types of energy in poor countries will occur relatively slowly and woodfuel will be dominant usage of harvested roundwood (FAO 2005a).

The trend in trades of tropical timber products undergoes some changing due to new policies. Generally, the export of industrial roundwood will decrease and the future will see higher export of processed primary (sawnwood, veneer sheets, etc.) and secondary forest products (furniture, packaging, etc.). There are several factors affecting the trend in exporting industrial roundwood: increasing domestic consumption in developing countries, reduced harvest levels due to environmental concerns, a shift towards higher-value processed products and marked reductions in demands in Asia (FAO 1999). Besides that, export ban and tighter controls imposed by Indonesia to regulate illegal trade since 2002 and diversion of roundwood to mills processing primary products such as in Malaysia have an effect as well (ITTO 2005). The importance of secondary processed products in the future is best reflected in the policies changes such as inclusion of these products by ITTO members in both the ITTO objective of further processing of tropical timbers and the Goal 1 of the ITTO Yokohama Action Plan providing for the organisation a strategy to undertake "regular assessment ... on secondary products" (ITTO 2005).

In future, tropical countries will most likely see an increase in wood production from secondary forests and agricultural plantation. Areas of secondary forests throughout the tropics are increasing dramatically, and in some tropical countries they now exceed areas covered by primary forests. Reliance on secondary forests is expected to increase as larger primary forest areas are most likely going to be designated as protection forests. Secondary forests are a good source of wood fibre, non-timber forest products, social and environmental services and other goods. Once the potential of lesser known species is explored and forest management starts to focus on these forests, wood production will shift gradually

from primary to secondary forests. In the Asia-Pacific region, agricultural and industrial crops are starting to be recognised as new source of raw materials for forest industries (FAO 2005a). Such crops are rubberwood (*Hevea brasiliensis*), coconut palm (*Cocos nucifera*) and oil-palm (*Elaeis guineensis*). For example, rubberwood is being processed into sawnwood and further into furniture. In addition, it is increasingly being used for production of particle board, medium density fibreboard, plywood and cement board. In Malaysia, 80% of furniture output utilises rubberwood and the exports of rubberwood products are valued at about US\$ 1.1 billion (FAO 2005a). It should finally be mentioned that also agricultural residues can be important substitutes for wood fibres (FAO 2005a).

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4. Forest Plantations

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Introduction

Disturbances are the main driving force of forest dynamics and often lead to stand regeneration via seedlings of the same species. Secondary succession, the establishment of woody and non-woody pioneer plant communities, can be observed after forest removal due to high intensity disturbances such as forest fires or hurricanes extending over broad areas (Kimmins 1996). Global tree harvesting systems such as clear cutting (Dohrenbusch 2001) or shelterwood systems over large area result in disturbances similar to catastrophic natural events. Tree planting is introduced to bridge the unproductive pioneer stages of forest develop-

ment, to regulate tree species composition, and to avoid possible site degradation processes such as erosion and nutrient leaching (Bormann & Likens 1979, Puhe & Ulrich 2001). Forest transformation is another focus of tree planting. The advanced planting of shade tolerant species such as European beech (*Fagus sylvatica* L.) under the canopy of conifers is promoted in Europe in order to increase the naturalness of the forest cover as well as to guard against abiotic and biotic hazards (Olsthoorn et al. 1999, Oldeskog & Löf 2005). The term **forest plantation**, according to FAO (2000), includes the establishment of forests through planting or seeding in the process of afforestation and reforestation. **Afforestation** is the direct human-induced conversion of land that has not been forested for a period of at least 50 years to forested land status through planting, seeding or the human-induced promotion of natural seed sources. **Reforestation** (or **reafforestation**) is the establishment of forest in areas forested through the previous 50 years in which either the previous crop is replaced by different species or by the same species as before. Both activities, reforestation and reafforestation, focus on indigenous and introduced species. Young natural stands and all plantations which have yet to reach a crown density of 10% or tree height of 5 metres are designated simply as 'forest'.

Historical and recent state of forest cover and forest plantations

Reforestation to compensate for forest removal is not a recent practice. Historical documents show that as early as the fourteenth century a large reforestation campaign was launched in the German Nürnberger Reichswald in anticipation of a firewood shortage (Hasel & Schwartz 2002). Overall, however, the number of such reforestation activities in historic times was limited and the reforested areas were generally small. The lack of consistent reforestation activities, coupled with both natural and human disturbances, resulted in the reduction of the forest land cover in Central Europe from more than 90% to less than 30% during the last 2,500 years (Küster 1995, 1998).

Global forest cover has been estimated at some six billion hectares prior to human impact. According to the 2000 Forest Resource Assessment completed by the FAO, the current global forest cover is about 3.869 billion ha. This equates to some 29.6% of the ice free land surface of the Earth. Of this area, approximately 95% is defined as natural forests. This includes planted forests with one or more indigenous tree species or forests with a heterogeneous composition. The remaining 5% of the current global forest area is comprised of what is referred to as 'forest plantations' having a uniform structure comprised, in the main, of introduced tree species (Table 1). The FAO Forest Resource Assessment from 2000 notes that almost 70% of global forest land is located in just ten countries.

Table 1 Forest land area change in the 1990s, divided into continents (FAO 2000)

Forest areas (1000 ha)	World	Africa	Asia*	N/Central America**	South-America	Europe**
Total forest area 2000	3 869 455	649 866	745 416	549 304	885 618	1 039 251
Natural forest area 2000	3 682 721	641 830	626 721	531 771	875 163	1 007 236
Plantation area 2000	186 734	8 036	118 695	17 533	10 455	32 015
Change in forest area (%):						
Total forest area 1990-2000	-2%	-9%	-1%	+1%	-4%	0%
Natural forest area 1990-2000	-4%	no data	-1%	+1%	-5%	+1%
Plantations 1990-2000	+3%	no data	+5%	+1%	+7%	0%

* Asia incl. Middle East, Australia and Oceania

** Europe incl. Russian Federation

From a climatic point of view, 47% of global forests are tropical, 9% are situated in subtropical zones, 11% are temperate, and 33% boreal forests.

Between 1960 and 1990, tropical forests declined by about 450 million hectares. During this thirty-year period, forests in Asia were reduced by 30% and in Africa and Latin America by 18% each. Maximum deforestation rates were registered in the 1980s when, in some years, the annual loss was as much as 20 million hectares. In the 1990s, the world-wide loss of forests, mainly in the tropics and subtropics, averaged 14.6 million hectares per year. Countering this, reforestation efforts were active on some 5.2 million hectares, primarily in temperate and boreal zones. The global net loss of forests, therefore, was some 9.4 million hectares per year (Mha yr^{-1}), a relative net change of -2.4% (FAO 2000). The majority of forest loss took place in the tropics with a net change of minus 12.3 million ha, compared to an increase of 2.9 million ha in the rest of the world. During this thirty year period, the FAO notes that forests disappeared completely in twenty-five countries while another twenty-nine countries lost more than 90% of their forested lands. Africa has suffered the most dramatic loss of forest land. Within the last decade of the twentieth century, the global forest area decreased by 9% (Table 1).

The forest plantation area was estimated to be 187 million hectares worldwide (Table 2, FAO 2000). In Asia and South America, the general trend of forest reduction is balanced by strong reforestation efforts. With relative forest land area increases of 5% in Asia and 7% in South America it would seem that reforestation efforts are similar. The absolute area of new plantations is, however, almost ten times higher in Asia. A small increase of forest lands has been noted in Europe, due primarily to afforestation activities focusing predominantly on 'natural forests'. The low amount of forest plantations in Europe and other industrialised temperate and boreal countries can be attributed, in part, to the definitions that classify most planted forests under management as 'natural forests' (FAO 2000).

New forest plantation areas are being established at the rate of 4.5 million hectares per year (Table 2, FAO 2000). The majority of this growth, 3.5 million hectares, occurred in Asia with China and India combined showing the largest relative growth. With a total plantation area of 45,083 ha, about 24% of global plantations have been established in China while India has the second largest plantation area with 32,578 ha. In the case of India, the plantation area represents half of the total forest area of the country and 17% of the world plantation area. Following China and India, are Russia, the United States, Japan, and Indonesia, each with a relative share of less than ten percent. These countries are followed by Brazil, Thailand, Ukraine, and Iran, each with between 1 and 3% of world plantation area. These ten countries represent 144 million ha, 80% of the total plantation area in the world. The main species and most common genera of forest plantations are *Eucalyptus* and *Pinus*. Few species of these two genera constitute about one third of the world's plantation area. *Eucalyptus* plantations are found on a large scale basis in India, Brazil, South Africa, and Vietnam. In Chile, Australia, South Africa, Brazil, and China, on the other hand, *Pinus* species are the most common plantation species. Other conifers comprise about 11% of global plantations while various unspecified species add up to nearly 30% of the total global forest plantations.

Though the yields from forest plantations depend on site condition, tree species, and sensitivity against pest and diseases, in most cases the productivity of plantations is higher than from natural forests. In the tropics, the annual plantation wood increment ranges between 10 and 30 m³ ha⁻¹ yr⁻¹ compared to 1 to

Table 2 Main species of global plantations (FAO 2000)

Table 2. Main species of global plantations (FAO 2006)										
Region	Total area (1000 ha)	New plantations (1000 ha ⁻¹ yr ⁻¹)	Plantation areas (1000 ha) by species groups							
			<i>Acacia</i>	<i>Eucalyptus</i>	<i>Hevea</i>	<i>Tectona</i>	Other broad-leaves	<i>Pinus</i>	Other conifers	Un-specified
Africa	8036	194	345	1799	573	207	902	1648	578	1985
Asia	115847	3500	7964	10994	9058	5409	31556	15532	19968	15365
Europe	32015	5					15			32000
N-/Central America	17533	234		198	52	76	383	15440	88	1297
S-America	10455	509		4836	183	18	599	4699	98	23
Australia/Oceania	3201	50	8	33	20	7	101	73	10	2948
World total	187086	4493	8317	17860	9885	5716	33556	37391	20743	53618

5 m³ ha⁻¹ yr⁻¹ from natural forests (Evans & Turnbull 2004). The *Eucalyptus*, on an annual basis, can yield as much as 45 m³ ha⁻¹ yr⁻¹ or more, while yields of up to 100 m³ ha⁻¹ yr⁻¹ have been recorded (Brown 1999). A focus on fast growing tree species such as *Eucalyptus* combined with fertilisation practices are seen as the underlying reasons for such high productivity of plantations. Compared to natural forests, plantations offer a more uniform timber quality which helps to forecast the future supply. The rotation period ranges between seven years for some *Eucalyptus* plantations in the tropics to more than 100 years in temperate and boreal zones (Savill et al. 1997).

Motivations for reforestation and afforestation

About 50% of the world's natural forests are not available for use (Fenning & Gershenzon 2002). In the face of a dramatically increasing world population, there is strong and increasing demand for wood even where the anticipated consumption per person does not change or decrease. There is evidence that the increase of wood demand seems to be higher than the population growth. Recently, world population has shown a 1.3% increase per year. This corresponds to an average increase of about one billion persons per decade with an estimated and predicted population of some nine billion by 2054. The global demand for wood, however, is increasing by 1.7% per year (FAO 2000). In 1960, the global average forest area was approximately 1.2 ha per capita. This ratio has dropped to a calculated 0.6 ha per capita in 1995. The predicted global average forest area for 2025 is 0.4 ha per capita. The FAO 2000 report indicates that the overall demand for wood is expected to increase by 25% from 1996 levels to just under 1.9 billion cubic metres in 2010. It was estimated in 2000 that more than 250 million people in some twenty countries did not have sufficient access to forests to meet their wood needs. By 2025, this number is expected to rise to more than 800 million persons.

Wood is the most important energy source for about three billion people. Over 50% of the world's harvest of wood is used as fuel (firewood and charcoal). More than 80% of this consumption, an increasing trend, takes place in developing countries. It is estimated that some 80% of the world's population does not have access to the minimum amount of paper considered necessary to fulfil basic needs in reading and communication. The lack of paper is a serious threat to the efficiency of educational programs in developing countries (Gardner-Outlaw 1999).

In 1992, the European Commission launched a program to increase afforestation activities on farmland. The purpose of the program was to reduce the costs of agricultural subsidies. Agricultural subsidies, at that time, were the largest single item in the EU budget. Landowners willing to convert agricultural land into forest production received afforestation grants which included a cost support for maintenance during the first critical years as well as forest premium compensation

for the income lost from agricultural products. Within the first decade of the program's launch, about one million hectares were afforested in the European Community, mainly in Spain, Portugal, and Ireland. Countries implementing this program were allowed some flexibility, within a limited framework, to modify the grants and premiums permitted. In Ireland, for example, afforestation grants differed between 2,000 Euro and 5,000 Euro per hectare, dependent on tree species composition. Plantations of conifers such as Sitka spruce (*Picea sitchensis*), for example, or lodgepole pine (*Pinus contorta*), with some 2,500 plants per hectare, attracted a grant of about 2,000 Euro plus 700 Euro for maintenance. For broad-leaved species, such as the common oak (*Quercus robur*) or the European beech (*Fagus sylvatica*), the afforestation grant was more than 5,000 Euro along with maintenance compensation of 1,600 Euro. A forest premium is paid up to 20 years for farmers but only 15 years for non-farmers. The amount of the premium is related to the proportion of broadleaves: approximately 300 Euro per year for pure conifer plantations and about 500 Euro for a new forest with deciduous trees.

Apart from the socio-economic impact attributed to reforestation and afforestation, it is suggested that there are also significant ecological benefits. Deforestation is the most important single cause of land degradation followed by agricultural activities and overexploitation of vegetation (Oldemann et al. 1991). The removal of forest is often followed by soil erosion. Worldwide, the total land area subject to human induced erosion is estimated at some two billion hectares (Oldemann et al. 1991). Land degradation was estimated in 1991 to be increasing by five to six million hectares annually. In 1996, land degradation had effected nearly 2,000 million ha (15%) of the world land area. Afforestation and reforestation are both contributors to a reduction in flooding, to the stabilisation of slopes, to the prevention of desertification, and to increases in the biodiversity of bare land. They also act as windbreaks and help to improve the global problem of climate change through carbon sequestration. Under good site conditions, carbon sequestration can increase in forest plantations since up to 8 tons C per ha are captured (Schlamadinger & Karjalainen 2000; see also Chapter 5 of this book). Due, however, to the subordinate proportion of plantations to total forest cover, a significant effect on the average global carbon sequestration rate cannot be anticipated. Assuming that the average carbon sequestration rate of the world's forests is about one ton per hectare annually, an optimistic estimation, it would be necessary to double current forest land area to capture the anthropogenic carbon dioxide emission of approximately seven Mt per year (Dohrenbusch 1998).

In the main, planting activities, particularly in developed countries, are undertaken to provide a continuing supply of timber and pulp. According to the FAO Forest Resource Assessment in 2000, 35% of the industrial roundwood came from plantation forest. This figure is expected to increase to about 44% in 2020. In countries where the pressure on natural forest by overexploitation is particular-

ly high, plantations can play an important role to reduce this form of ecological mismanagement (Savill et al. 1997, Brown 1999, Evans & Turnbull 2004).

The role of forest plantations in the Kyoto Protocol

One outcome of the United Nations Conference on Environment Development (UNCED) in 1992 at Rio de Janeiro was the agreement of 178 nations to a model of sustainable development, including the Forest Principles. At a national level, each of these countries were obligated, through their agreement, to create a National Forest Program (NFP) that included the certification of forest management and the conservation of forests as well as various reforestation activities. Since 1997, the Kyoto Protocol has focused on the effects of forests on a global scale, generally from the perspective of carbon sequestration. The Protocol required an average reduction of carbon dioxide and other greenhouse gases of 5% by 2010 relative as compared to 1990 levels (European Union -8%, Germany -21%, USA -8%, Japan -7%). According to the Protocol, carbon sources include all processes where carbon dioxide is emitted into the atmosphere as a result of changes in land usage. Carbon fixing activities, on the other hand, are regarded as a carbon sink. Not all parties to the Kyoto Protocol agreed on the definition for afforestation, reforestation, and the evaluation of carbon sequestration by forests and plantations. The main controversy coming out of the Kyoto Protocol, and a major concern of the United States of America, was the level or extent of existing forests. A United Nations Framework Convention on Climate Change was convened in The Hague (Den Haag), Netherlands in November, 2000 with the express purpose of developing and agreeing to additional details of the Kyoto Protocol as well as specifying implementation procedures for the Protocol. U.S. negotiators proposed that almost 310 million tons of carbon (about half of the U.S. reduction target) can be accounted for by the uptake of carbon from its forest and soils. The European Union as well as observers from some environmental groups refused to acknowledge this kind of sink because it does not require human activity. This disagreement is a controversy among some nations as well as a conflict between different economic lobbies. Representatives of emission-intensive industries and the Organization of Petroleum Exporting Countries (OPEC) favour the full acceptance of forest conservation and afforestation. The conference results have been disappointing to date inasmuch as the United States of America withdrew its support for the Kyoto Protocol in April, 2001 (for further reading, see Chapter 5 of this book).

Risks and perspectives of new forest plantations

A basic principle of sustainable forest management is the requirement to establish new forests on sites where former forest stands have been harvested. This is an essential rule in the forest laws and management plans of many countries. There

are, however, critical reservations with respect to the benefits of afforestation as a forest sink emissions compensator inasmuch as afforestation is seen as a form of damage mitigation rather than a solution to the problem of air pollution. With respect to the biodiversity of forests, the process of afforestation is often regarded as controversial since, while many ancient biodiverse woodlands are being lost, the replacement plantations are mostly monocultures. This is especially a problem in the tropics and subtropics where monocultures or plantations with a very limited variety of tree species often replace highly diverse ecosystems. There are relatively few examples where indigenous tree species are selected for forest plantations. This is unfortunate as it can be shown that for some site conditions efficient native tree species can reach almost the same productivity as the generally favoured exotic fast growing trees which are often chosen to replace what has been harvested (Arias & Dohrenbusch 2001).



Fig. 1 Afforestation plots with different clones of poplar hybrids in Germany

Countries with naturally high percentage of forest land may consider landscape aesthetic problems in the context of strong afforestation and reafforestation activities. In a Finnish study, the visual impacts of gradual afforestation were evalu-

ated by private non-industrial forest and agricultural landowners, potential recreational users, and land use management professionals. In general, the scenic beauty decreased with increases in the intensity of afforestation. An exception to this was moderate afforestation which can have a positive effect on scenic beauty. The more attractive the original landscape, the greater was the effect of afforestation. The perceived evaluative differences of landowners were not as distinct as those of the other groups. Attitudes towards afforestation were more positive compared to the perceptions of the other groups (Tahvanainen et al. 1996).

There is some general criticism that many plantations have been established on land claimed by indigenous peoples (Sargent 1992). Sedjo (1999) has shown that there are a growing number of examples from different parts of the world where the establishment of plantations are a result of the interests, almost always economic, of outsiders and where there has been limited consultation with local people. Tyyneä (2002) has reported a case study from Zimbabwe which details the inability of traditional users of non-timber forest products to access plantation forests to continue their previous harvesting practices. In general, this is because previously biodiverse forests have been replaced with monoculture growth.

Common planting techniques

In most parts of the world, particularly in the tropics and subtropics, the usual plantation technique is the use of young seedlings in plastic containers. Black plastic bags, perforated at the base, with volumes between 300 and 1000 cm³, are particularly used for conifers. After planting, the bags can be reused several times. In the temperate zones, however, especially in Europe, the planting of bare-rooted young trees has a long tradition. Under suitable site conditions, topography being a general limitation, planting machines can significantly increase the efficiency of the planting process. For large areas with limited infrastructure, afforestation by aerial sowing can be an economic alternative. For the Green Great Wall in China, a more than 4,000 km long shelterbelt from the northwest to the northeast, the total area afforested by aerial sowing has risen to 8.68 million ha. In arid zones, in particular on karst sites, success rates are improved with a variety of planting aids. A frequently used method is covering the soil around a newly planted sapling with small stones so as to reduce evaporation. Soil filling material can be mixed with small stones or pebbles to interrupt soil capillarity. This method avoids a continuous water stream from deeper parts to the surface. Even more effective, but more expensive, is the application of hydrogels (Hüttermann et al. 1997; see also Chapter 5 of this book).

Where forest plantations are endangered by grazing animals, protection measures are necessary in the early phases of growth. This can be accomplished by fencing the entire plantation area or through individual tree protection. Mechanical methods are often used such as protecting the tree with the branches of spiny

shrubs or the use of metal lattices or plastic tubes. Chemicals may also be used to protect the top buds of the young trees. The protection from small rodents (mice), which can cause enormous damage by destroying the root system of new plants, is often difficult. In the northwest of China, for example, because there are limited options for pest control, mice populations have damaged large afforestation areas (Dohrenbusch, own observation).

Conclusions

Up to 15 millions hectares of forests are still destroyed worldwide though the annual loss of forest area decelerated. On the other hand, afforestation activities of approximately 5 millions hectares, particularly in China and India reduce net deforestation to 10 million hectares at a global level. These countermeasures can however not replace the loss of predominantly primary forests. The restriction on few tree species usually with above average production, the predominant use of non-domestic, imported varieties on plantations with comparatively short production cycles point out the problem: Forest plantations are no alternative for natural forests. They can however contribute to regional wood supply and carbon sequestration. Their importance will continue to increase in the future. Nonetheless, the primary goal cannot be an expansion of compensatory afforestations and reforestations but a strong reduction of forest loss worldwide.

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5. Carbon Dioxide, Forests, and the Kyoto-Process

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Carbon Dioxide and Climate Change

A possible fast climatic change has become an important issue of scientific discussion (e.g. Anonymous 2002a, Kohl & Kühr 2004). In view of the very complex interactions between the anthropogenic emissions and the climate of the world and in order to avoid too much controversial discussions within the scientific community and to provide solid information for the necessary political decisions, the WMO (World Meteorological Organization) and the UNEP (United Nations Environment Programme) established in 1988 the IPCC (Intergovernmental Panel on Climate Change; <http://www.ipcc.ch/index.html>).

The task of the IPCC is to review all available scientific, technical and socio-economical information pertinent to the topic “climatic change” which is pub-

lished in the peer-reviewed literature and to reveal the results in form of reports. The first report appeared in 1990 (Houghton et al. 1990, Tegart et al. 1990) and served as the scientific basis for the Rio-Conference in 1992 where the Agenda 21 was passed by the governments of the world. There is now an almost unanimous agreement in the scientific community that the increase in carbon dioxide concentration of the atmosphere is mainly responsible for the observed global increase in the temperature of the earth and that the global warming has to be reduced or at least the increase to be stopped (Forrest 2005).

The Rio-Conference was followed up by several other conferences in which special problems were discussed. The conference in Kyoto (1997) focused on the greenhouse gas, especially the carbon dioxide problem. In the Kyoto-Protocol, the governments of the world agreed upon a massive reduction of the present greenhouse gas emissions. This protocol is now ratified by most of the governments of the world, except the USA, Australia and a few other countries. It is now in force as a law in the European Union (EU; http://unfccc.int/essential_background/kyoto_protocol/items/3145.php).

For the implementation of the Kyoto-Protocol, the United Nations Framework Convention on Climate Change (UNFCCC, Bonn) was established, an agency which at present elaborates the rules for the assessment of relevant projects (<http://unfccc.int/2860.php>).

Strategy presently envisaged for the mitigation of the carbon dioxide-problem

The joint science academies' statement "Global response to climate change" (Anonymous 2005) considers the increase in the carbon dioxide concentration in the world's atmosphere to be the most important factor for this threatening climatic change and asks for the reduction of the causes of the climate change: the emission of carbon dioxide into the atmosphere.

In spite of the fact that the Kyoto-Protocol has not been ratified by the USA (http://unfccc.int/essential_background/kyoto_protocol/items/3145.php), enormous amounts of research money are presently invested in the research for solutions of the "carbon dioxide dilemma". The National Academy of Engineering organised together with the National Research Council of the National Academies of the USA in April 2002 a symposium, on which relevant aspects were discussed. The results of the symposium were published in the following year (Anonymous 2003a) and became a master plan for future strategies in CO₂-reduction. The strategy proposed by this symposium is the following:

1. Removal of the carbon dioxide from the flue gases of the power plants in which they are generated: The flue gasses are injected into a solution of monoethanol amine which removes about 90% of the carbon dioxide from the

gas stream. The monoethanol amine-carbon dioxide complex, which is formed during this reaction, is then processed in such a way that the monoethanol amine is regenerated and the carbon dioxide is recovered. After the chemical isolation, the CO₂ is liquefied under a pressure of 110 bars (Anonymous 2003a).

The capacity of a typical power plant is about 600 MW. It produces about 500 t carbon dioxide per hour. For the absorption of this amount of carbon dioxide, about 16,000 m³ of monoethanol amine have to be circulated. The carbon dioxide absorption reduces the efficiency of the power plant by 10-15 percent points (which in a modern plant with an efficiency of 50% corresponds to a reduction in the electricity production of 30%). The carbon dioxide absorption requires a doubling of the investment costs. The cost of the carbon dioxide production under such conditions is estimated to be in the range of 45 \$/t CO₂ - this price has been declared as the target for a R&D (Research & Development)-project on sequestering CO₂ from flue gases that is carried out at present by the Lurgi AG, Frankfurt, Germany (Plass 2004).

2. Transport to the place of deposition: In the liquid state, carbon dioxide can then be transported either in special pipelines or in tanks on trucks or ships. In construction, a CO₂-tank-ship will be similar to the transport ships for LPG (liquefied petrol gas). The first prototype is already in operation. Depending on the distance and the means employed, the costs for the transport will be in the range of 10-15 \$ per ton of carbon dioxide (Anonymous 2003a).

3. Deposition of the carbon dioxide somewhere: The following options of carbon dioxide sequestration are under discussion and the target of rather intensive research (Anonymous 2003a):

- Direct disposal into the ocean
- Sequestration via injection of carbon dioxide into the deep earth
- Using carbon dioxide to recover natural gas and oil
- Geologic sequestration of carbon dioxide

The costs for the deposition are estimated to be in the range of 10 \$/t carbon dioxide (Anonymous 2003a).

This three-divided concept developed at the 2002 symposium is the blue-print for virtually all research activities pertaining carbon dioxide mitigation. In 2003, the ministers responsible for energy of the OECD (Organisation for Economic Co-operation and Development) and some threshold countries agreed upon the first international treaty on carbon dioxide sequestration. In Germany, the COORETEC-Program (CO₂ Reduktionstechnologien – CO₂ reduction technologies; http://www.cooretec.de/index_cooretec.php) was started, which funds research on the technical sequestration of CO₂ and its geological storage (Anonymous 2002b). The same line of thinking is followed by the German “Rat für Nachhaltige Entwicklung – Council for Sustainable Development”

(<http://www.nachhaltigkeitsrat.de/>) in its recommendation on strategies for carbon dioxide-free coal power plants (Anonymous 2003b).

To summarise this concept of CO₂-reduction strategies, two points are very clear:

1. This option is pretty expensive. Howard Herzog from the MIT (Massachusetts Institute of Technology) in his closing words of the conference of the National Research Council of the National Academies of the USA in 2002 discussed final prices in the range between 100 and 200 \$ per ton of sequestered carbon or 30 – 60 \$ per ton of carbon dioxide (Herzog 2003).

2. The best what can happen to the sequestered carbon dioxide is that it stays at the place where it is finally stored. It will be not useful for anything else.

Biological carbon sinks – the neglected alternative

The “Fachgruppe Umweltchemie und Ökotoxikologie – Section Environmental Chemistry and Ecotoxicology” (<http://www.gdch.de/strukturen/fg/uoef.htm>) of the “Gesellschaft Deutscher Chemiker – Society of German Chemists” has published a position paper, which was officially adopted by the Governing Board of the Society. This position paper states that the chemical-technical solutions for the sequestration of carbon dioxide are a wrong way of coping with this problem and that the terrestrial biosphere is the most efficient system for sequestering CO₂ which has shown its reliability by now for millions of years (Anonymous 2004; Hüttermann & Metzger, 2004). The ecological basis for this statement becomes evident by studying Fig. 1. Here, the monthly measurements of the carbon dioxide concentration in the ambient air at the measuring station on the Mauna Loa Mountain on the island of Hawaii are given. These data very clearly document that the mixing of the gases in the atmosphere is so fast, that the growing season of the forests of the northern hemisphere can be recorded on a station which is located more than 4,000 km away of the nearest of these forests (Keeling & Whorf 2005).

With the atmospheric CO₂ concentration data at the Mauna Loa Mountain, Keeling et al. (1996) further demonstrated that the seasonal carbon dioxide cycle changed its pattern. The yearly amplitude has increased (Fig. 1) and, between the early 1960s and the 1990s, the phase has advanced by about 7 days during the declining phase of the cycle. From these data it is evident that the circulation of the earth's atmosphere is so fast and efficient that any carbon dioxide which is added at some place in the world is distributed over the whole globe within a short time. The same reasoning holds true, of course, for the removal of carbon dioxide. It does not matter where a sink for carbon dioxide is established - its effects will reach within a short time the whole globe.

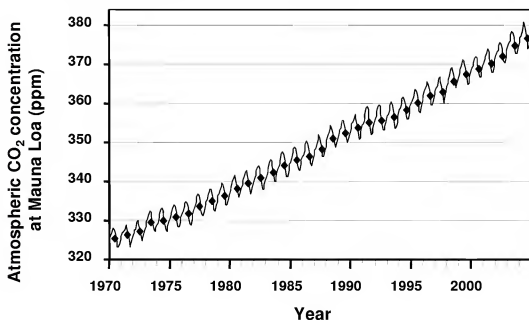


Fig. 1 Monthly mean values of atmospheric CO₂ concentrations (in ppm) measured at the Mauna Loa Observatory, Hawaii for the period 1970 to 2004. Seasonal fluctuations are related to photosynthesis (spring, summer) and the decomposition of organic carbon (autumn, winter). Diamonds indicate the annual average of the atmospheric CO₂ concentration (after Keeling et al. 1996 and Keeling & Whorf 2005)

Carbon binding potential of forest ecosystems

The potential of afforestations for carbon dioxide sequestration is presented in Table 1. Under European conditions, fast growing tree species like willow (Christersson 1986), poplar (Heilman & Stettler 1984) or grand fir (Kramer 1984), produce about fifteen to twenty tons of round timber per ha. This amount of timber is equivalent to 27–36 tons of carbon dioxide. In addition to this sequestration in the form of timber, a stand absorbs much more carbon in the form of leaves, twigs and humus. A balance of the total carbon dioxide binding was made for an afforestation project financed by the World Bank in the frame of the Prototype Carbon Fund (Brown et al. 2002). From these data it is obvious that an afforestation with fast growing tree species has a much higher efficiency in carbon dioxide sequestration than annual agricultural crop plants could ever have (see also Chapter 4 of this book). The question which then arises is: How much land would be available for such afforestations without impeding the production of food and fodder?

At present, about 26 billion tons carbon dioxide annually are worldwide emitted. To sequester this amount by afforestation, one would need 480 million ha tropical dry forest or 3 billion ha desert area, given the present values of biomass

Table 1 CO₂-Sequestration potential of afforestations in different regions of the world

Forest type	Tons CO ₂ /ha/a in the biomass	Tons CO ₂ /ha stored in the soil	Reference
Tropical dry forest	55	90	Tiessen et al. (1998)
Bamboo forest	47	100	Hui & Yang (1998)
Short rotation plantations, Europe	37	1.000	Heilmann & Stettler (1984)
Afforestation in deserts	9	90	Watson et al. (2000)

production for the different regions. In the last century alone, about 1 billion ha of land has been severely degraded by converting tropical forests into not very fertile agricultural soils which eventually lost their fertility. During the same time, the Sahara desert has spread by about 100 km to the south which converted an area of about 520 million ha land into a desert. In addition, massive soil degradation has taken place owing to inadequate agricultural techniques which afflicts about 1.1 billion ha land (Lal 2004). In view of the modern afforestation techniques, which will be outlined below, there is no reason to believe that these degraded soils cannot eventually be afforested again (see also Chapters 3 and 4 of this book).

From the facts discussed above it is evident that biological carbon sequestration is the economic way to mitigate the carbon dioxide dilemma. In addition, it is obvious that the countries of the "Third World" are those which would be able to convey most of the land which is necessary for this process. The afforestation of these lands will be a basis for their own development (see also Chapter 3 of this book).

Afforestation of degraded lands

The experience which was made during the last fifty years by Keren Kayemeth LeIsrael (KKL, http://www.kkl.org.il/kkl/kklmain_blue_eng.aspx), the Jewish National Fund, with regard to desert afforestation in the Northern Negev in Israel is really encouraging. The established practice of the Forestry Division of KKL begins with a large scale preparation of the land. The hill slopes are terraced with an earth mover. At the beginning of the winter – the rainy season – one year old trees are planted with conventional methods. A method for helping the sapling to survive the first harsh summer is the use of plastic tubes in which the planted trees are inserted. After the first year, the tubes are collected and used again.

The usual strategy of afforestations in the Northern Negev is to plant the trees on the hill tops, the valleys are left free for agricultural settlements. After a stand age of about 30 years, the hydrological conditions have developed in such a way,

that irrigation agriculture can be performed with significantly less water than in the case of barren hills. In the Lahav and Yatir Forests several agricultural cooperatives (Moschavim and Kibbutzim) have settled there meanwhile for several decades with good performances with regard to the overall economy of their ventures (http://www.kkl.org.il/kkl/kklmain_blue_eng.aspx).

Although the success of Keren Kayemeth LeIsrael with regard to desert afforestation is really stunning, not all stands are suitable for the techniques the Israelis are using. Also, not for every stand a suitable novel tree can be found which is able to grow on it right away. However, we discovered that stands where trees fail to grow in many cases can be afforested with the help of hydrogels (Hüttermann et al. 1999).

Hydrogels in general are by now an almost integral part of our modern culture. They are the „miracle substances” in the diapers which keep the babies dry for the whole night. Hydrogels are crosslinked polyacrylates of very high molecular weights. These substances very fast bind water, up to 400 times their weight. This rapid and reversible absorption of water will take place also when the hydrogel is dispersed in soil. When the soil starts to dry out, the water which is stored in the hydrogels is helping the plant to survive the drought (Hüttermann et al. 1999).

The most comprehensive field experiments with hydrogels so far have been conducted by Prof. Ma Huancheng, Southwest Forestry College Kunming, in the eroded areas of the drainage basin of the upper Jangtze river in Yunnan, China. The survival of the plants treated with hydrogels was twice as high as of the plants cultivated without the hydrogel. An amendment of the soil in the plant hole with 40 g hydrogel was enough for the survival of the trees. These results were confirmed in the meantime for other tree species in other parts of the hot dry valleys (Fig. 2; Ma & Nelles-Schwelm 2004). Based on the results of the afforestations employed so far, it is now planned to until 2010 afforest several hundred thousand hectares of degraded lands all over China (Ma, personal communication).

What is the price for carbon dioxide sequestration in an afforestation?

The Kyoto-Protocol includes afforestation and the subsequent use of the wood as a sink for reducing the carbon dioxide content of the atmosphere. Thus, a “pure” afforestation could lead to a profit, if the carbon dioxide which is bound in the standing timber could be certified and these certificates sold accordingly.

Fig. 3 shows that the price for the ton of carbon dioxide sequestered by afforestation is a clear function of time. The longer the forest is allowed to grow, the lower the price for carbon dioxide sequestration will be. This is true, however, only for the first 20 years of stand age. Owing to the imputed interest – in this calculation set to 5% - the prices increase again after 20 years.

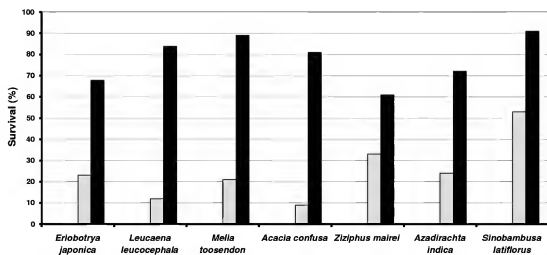


Fig. 2 Results of the afforestation experiments in the Hot Dry Valleys in Yunnan. Grey bars: controls; black bars: 20-40 g hydrogel per plant pit (Ma & Nelles-Schwelm 2004)

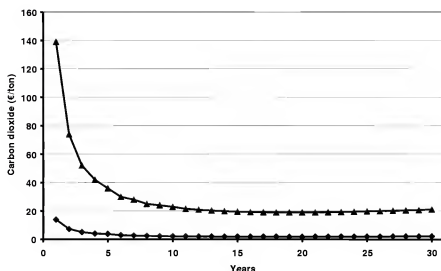


Fig. 3 The costs for the binding of one ton of carbon dioxide in the form of standing timber; stand productivity: 20 t/ha/a. Upper curve calculated for German prices and wages (5,000 € for afforestation, 90 € for maintenance), lower curve for Chinese prices and wages (500 € for afforestation, 3 € for maintenance). Imputed interest: 5%

A comparison between the development of the price for carbon dioxide (Fig. 4) and the net discounted revenues for afforestations (Fig. 5) reveals that only under Chinese economical conditions it will be possible to grow forests exclusively for the purpose of carbon dioxide sequestration. An investment of

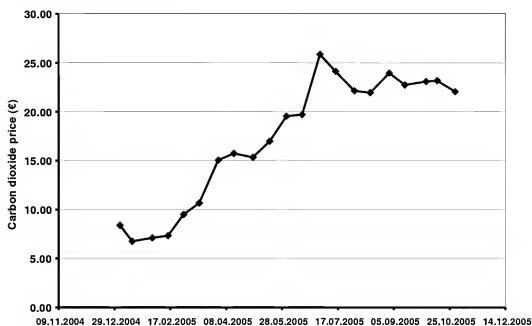


Fig. 4 Development of the price for carbon dioxide at the European Energy Exchange, Leipzig (http://www.eurexchange.com/about/company_info/subsidiaries/sub_eex.html)

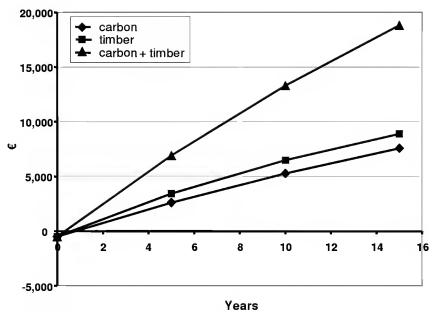


Fig. 5 Net discounted revenues for afforestations under Chinese economical conditions (cf. Fig. 3) with and without a carbon dioxide-bonus. Parameters: costs for afforestation: 500 €/ha; timber yield: 20 t/ha/a, i.e. 36 t CO₂/ha/a; costs for maintenance: 3 €/ha/a; revenue: 50 €/t round wood; carbon dioxide sequestration: 20 €/t of CO₂; imputed interest: 5%

500 € will lead to a net discounted revenue of 7,000 €, which is an internal rate of return of about 90% (Fig. 5).

This way of achieving income, however, will not be possible in the biggest region of the world that so far has implemented the Kyoto-Protocol as a law, i.e. the EU. „Pure“ afforestation is excluded from the certifying process in the EU. The reason for this is given very clearly in the pertinent EU-document (Anonymous 2003c; abbreviations: JI = joint implementation, CDM = clean development mechanism - for further details see articles 6 and 12 of the Kyoto Protocol):

“3.2 Qualitative Conditions:

JI and CDM credits that may be generated through land use, land use change and forestry (LULUCF) activities are also excluded from recognition. LULUCF activities can only temporarily store the carbon, which will at some time be released into the atmosphere. They are not covered by the Community emission allowance trading scheme, which aims at achieving permanent reductions from emission sources. The Community trading scheme is very much designed as a technological driver for long term emission abatement improvements from energy and industrial sources. Recognising credits from LULUCF activities would not be consistent with the approach taken by the Council and the European Parliament on emissions trading. Furthermore, there are still many uncertainties as to how to account for and monitor emission removals by sinks under the Kyoto Protocol, both under JI and the CDM, at country- and at project-levels. It is not clear how the temporary and reversible nature of LULUCF carbon sequestration can be reconciled with entity-level emissions trading, as this would have to involve the attribution of subsequent releases of greenhouse gases to the beneficiary from the initial sequestration. Negotiations are currently in progress for the design of modalities for the inclusion of afforestation and reforestation under the CDM, and these will not be agreed internationally before the 9th Conference of the Parties to the UNFCCC (December 2003) at the earliest. In the light of the application of these modalities, the Commission will give due consideration to whether and, if so, how credits from LULUCF activities could be used in entity-level emissions trading in the Community scheme. In addition, the JI and CDM should bring technology transfer through, for example, the promotion of new, cleaner technologies and improvements in energy efficiency, while afforestation and reforestation activities do not bring technological transfer or development. Because sinks projects are expected to be cheaper than projects involving the transfer of technologies, allowing credits from such projects to be converted would be at the expense of promoting technological transfer to other industrialised and developing countries which is key to the JI's and CDM's success and the long-term goal of stabilising global levels of greenhouse gas emissions.” [Quoted from the EU-Decision (Anonymous 2003b)].

This statement can be summarised as follows:

- Afforestation is – rightly – considered as a method of carbon dioxide sequestration which is much cheaper than all other technological solutions discussed above.

- Afforestation or reforestation (of stands which have been clear cut decades ago) as such will not lead to the promotion of “technological transfer to other industrialised and developing countries which is key to the JI’s and CDM’s success and the long-term goal of stabilising global levels of greenhouse gas emissions.”

An inspection of the web-site of the CDM-office in Bonn (UNFCCC; <http://unfccc.int/2860.php>) shows that so far no single afforestation project has been accepted. The difficulties connected with the recognition of afforestations as a certified means to store carbon are the following uncertainties (Dr. Fuentes, German Ministry of Environment, personal communication to the authors):

- Permanency of the carbon binding over centuries in undisturbed forests
- Possible instabilities of forests: burning, ecosystem degradation owing to climate changes
- Problems with methodologies: monitoring, establishment of a reference site, etc.

Therefore, it is not likely that large scale afforestation as such, without a use of the product which is relevant to carbon dioxide mitigation, will be certified anyway.

How can “Third World” countries participate in the Kyoto-Process?

For “Third World” countries, meaningful participation in the Kyoto-Process would be to establish a sink for carbon dioxide via afforestation with subsequent conversion of the biomass to electrical energy and/or liquid fuel.

This approach would indeed result into a double income from the produced timber: first from the sale of the product and second for the carbon dioxide sequestration. The return of such a venture would be about 240% annually with regard to the invested capital (Fig. 5).

The following options exist for utilising the such produced timber in a way that is compatible with the Kyoto-Protocol and certifying process:

- Electrical energy: Alstom Germany has built in the last five years four 20 MW-power plants based on the combustion of wood in Germany. For the electricity production, they use 125,000 t per year of air-dried wood (<http://www.de.alstom.com/home>).

- Conversion of the wood to charcoal: In the Prototype Carbon Fund project: "Plantar Sequestration and Biomass Use" (De Ferranti et al. 2002), it is planned to plant in Brazil 23,000 ha of high yield clones of *Eucalyptus* spec. in order to produce charcoal for the pig iron manufacture.
- Conversion of biomass to chemical feedstock and synthetic fuels: The conversion of wood to diesel or methanol is the most efficient way to produce chemical fuels from biomass. A small plant would require about 300,000 t of timber per year to produce 150,000 tons of methanol or 75,000 tons of diesel oil (Plass & Reimelt 2007; see Chapter 6 of this book)
- Burning at high temperatures in high-tech furnaces to process either cement, lime, tiles, ceramics, or ore: An average German cement plant needs about 1.4×10^6 GJ energy per year. To supply this energy via afforestations, one has to produce about 366,000 t of dry woody biomass per year (Marton & Alwast 2002).

Conclusions

An afforestation in "Third World" countries as depicted above with subsequent conversion of the major part of the biomass to fuel would be beneficial to the world in several ways:

- Carbon dioxide would be removed from the atmosphere.
- Fossil fuel would be substituted with commodities from renewable resources.
- The project would be a model for a sustainable economy. It would show that it could be possible to establish a sustainable world economy without drastically changes in the machinery which is needed for our present way of life.

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6. Wood as Renewable Energy Source

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Introduction

Costs of raw oil in the last decade and prognosis for the next years support the expert thesis of the end of increasing rates of oil production at low costs. Consequences on the overall production and economy running mostly fossil fuels are obvious. Increase in demand on energy by developing countries is likely to generate a permanent boost of oil, gas, and coal costs on the world market (EIA 2005). Although searching for alternatives started long ago, there is at present not the faintest indication for an effective new system based on totally different materials that could fundamentally substitute fossil fuel in our present civilisation (Johansson 1999, Wüst 2006).

Environmental concerns related to greenhouse effect and global warming argue for no use at all of fossil fuels and, instead, for developing and applying of

cleaner and renewable energy sources (RES). European Union (EU) countries have committed themselves to reduce the emissions of greenhouse gases in the Kyoto Protocol commitment period 2008-2012 by 8% of the level from 1995 (http://unfccc.int/essential_background/kyoto_protocol/items/3145.php). As a consequence, the EU plans to double the fraction of renewable energy production from 1995 to 2010 and to triple the share of bioenergy in this time (Anonymous 1997). This impact in new technologies and industries is expected to generate 900,000 additional jobs in the EU, with 50 to 90% of them in usage of biomass fuels (Domac et al. 2005). The renewable energy industry is already now one of the fastest growing sectors. Employment related to production of energy from biomass such as bio-ethanol and wood fuel in developing countries reach already 1 million in Brazil and 3-4 million in India (Domac et al. 2005).

To run our economy in the future with RES, we should first consider the basic economical data of the different options which we have at present:

- Photovoltaic energy
- Solar energy for heating
- Wind energy
- Water power
- Biomass

The alternative energy sources are not easy to compare and such comparisons can hardly account for the future developments and innovations that are to be expected in next decade and that can strongly improve effectiveness and costs calculations (Goldemberg & Teixeira Coelho 2004, Wüst 2006). However, what we can see now are the considerable differences in their basic economy (Fig. 1).

The choice between the available options of RES is strongly dependent on local conditions, such as climate, type of agriculture, soil quality, cultural traditions, and economical demand and potential (Balat & Ayar 2005, Demirbaş et al. 2004). Bioenergy based on wood utilisation and afforestation with fast growing tree species is at the moment by far the cheapest way of energy production and the most promising RES for USA, Europe and Russia (Fujino et al. 1999). Non-biological but also renewable and easy to use photovoltaic technology is about 2.600 times more expensive (Fig. 1).

Concerning the biomass utilisation and the global carbon cycle (Fig. 2), several facts are of great importance for the installation of a sustainable energy supply: 80 to 90% of the living terrestrial biomass consists of trees (Watson et al. 2000). This biomass binds annually about 60 billion tons carbon dioxide. The system is in an equilibrium state, the same amount is released yearly owing to humus disintegration and wood degrading fungi (Nabuurs & Schelhaas 2003).

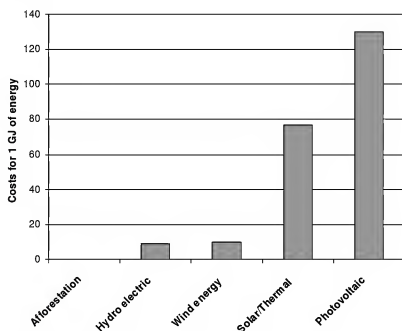


Fig. 1 Total costs for different means of production of 1 GJ of energy from renewable resources [van Bergen 2003; afforestation costs of 0.05 €/GJ are the author's calculations; photovoltaic: calculations were made by the German power company Energie-ag Mitteldeutschland (EAM; <http://www.eam.de/>), Kassel, Germany]

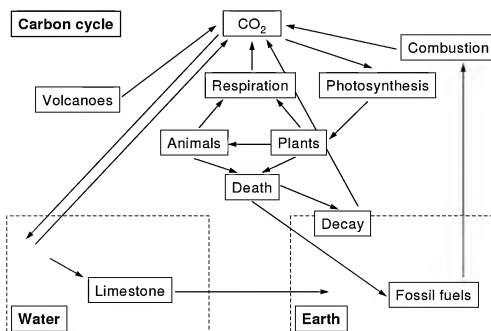


Fig. 2 Schema of global carbon cycle

Our economical activities are accompanied with a release of worldwide seven billion tons of carbon annually, which correspond to 26 billion tons of carbon dioxide. The terrestrial biomass is presently not able to absorb these additional amounts of carbon dioxide and this leads to the observed increase of this greenhouse gas. The carbon which is released into the atmosphere corresponds to about 8.3 billion tons of mineral oil (Aldy 2006, Marland et al. 2006).

The optimal design for new sustainable economy would be to use the present machinery (electrical appliances, cars and ships) with an energy base which will be more or less identical in its properties to the one which is used at present. This will avoid very costly switches in the basic machinery from one energy system to a completely different one. This “new energy” should be available in quantities similar to the ones which are used today. It is obvious that such an economy must be based on renewable energy sources.

Renewable Energy Sources

Our economic system is characterised by the fact that we are at the present almost totally dependent on fossil fuels as chemical energy carriers. With these, we transport our goods as well as ourselves on roads and railways, over the water and in the air. In addition, most of the electricity we are using is made from this type of chemicals also. Fuels have the following characteristics (McNeill 2003):

- They have a high energy density.
- They are easy to store and to transport.
- They are the basis of almost all the sophisticated machinery on which our life depends.

All these must apply also to the biomass as RES, if this should be utilised in the global scale and withstand the competition pressure of fossil fuels (Gan & Smith 2006).

Biomass in the sense of RES is generally defined as cellular material of living or dead organisms. It includes forest biomass as well as residues from forest industries and recycled wood, energy crops, agricultural residues or agrofood waste, manures and biogas, sewage sludge and the organic part of municipal waste. Biomass energy research performed in the United States and EU-countries concentrated mostly on trees (*Salix*, *Populus*), traditional row crops (*Zea mays*, *Triticum*), and rhizomatous grasses (*Panicum*, *Miscanthus*). Out of these, fast growing trees and grasses seem to be the most promising (Berndes et al. 2003). Based primarily on the moisture content, biomass can be divided into two groups: “dry” biomass comprising woody plants and materials, herbaceous plants and grasses, and “wet” biomass including water plants, manures, sludge, organic and food wastes. Due to high energy requirement for drying, the high moisture content of “wet” biomass routes its utilisation to aqueous conversion processes such as microbial fermenta-

tion, while “dry” biomass can be more economically applied for gasification, pyrolysis or combustion. Besides the moisture content, further characteristics of biomass, depending on the selected energy conversion technology, must be considered: caloric value, proportion of fixed carbon and volatiles, ash/residue content, alkali metal content, and cellulose/lignin ratio. Many of the biomass sources for energy conversion have a good potential for energy production but their availability is just limited or they are applicable as a local solutions only, e.g. sludge/biogas, agrofood waste, and organic municipal waste. Among the biomass resources with a potential for large scale (global) applications are cultivated plants. The most important parameter in overall comparison of such types of biomasses is the quantity of dry matter of biomass that can be produced from one unit of land area (McKendry 2002a). This value measured in dmt (dry matter tons)/ha combined with the so called higher heating value (HHV) represents the amount of energy that can be recovered from 1 ha of land within one year. The HHV in this calculation stands for the total energy content that can be released by burning of the material and therefore denotes the maximum amount of potentially recoverable energy.

A comparison of energy yields for selected biomass is given in Tab. 1. However, rural soils are usually much more fertile than the typical soils historically left for forestry by failure to be productive in agricultural cultivations even by medieval standards (Küster 2003). In realistic expectations on energy yields from biomass of agricultural crops and grasses, from plantations of fast-growing trees such as poplar and willow on fallow land, and from wood biomass obtained from typical forestry, land soil as well as climate conditions have to be considered (McKendry 2002a). Fast growing trees and rhizomatous grasses are considered as best options for production of energy from biomass in temperate climate areas (e.g. northern parts of the USA and the EU, Canada and China). In contrast for Eastern Europe and Central Asia, by poor soil conditions biomass production from woody plants and grasses is estimated to be generally very meager with values as low as 1 dmt/ha/a (Fischer et al. 2005; see Chapters 2 to 4 of this book for further information).

Table 1 Yields of energy from biomass under optimal growth conditions (McKendry 2002a)

Biomass	Crop yield (dmt/ha/a)	HHV (MJ/kg dm)	Energy yield (GJ/ha/a)
Wheat	14 (grain, straw)	12.3 (straw)	123
Poplar	10-15	17.3	173-259
Willow	10-15	18.7	187-280
Switchgrass (<i>Panicum virgatum</i>)	8	17.4	139
Giant Miscanthus (<i>Miscanthus x giganteus</i>)	12-30	18.5	222-555

Comparison of the different producers of biomass

Increasing the biomass of trees, woody plants and grasses is nowadays a subject of intensive studies. Selection of fast growing, effective species fitting to respective climatic requirements is accompanied by production of hybrid plants and genetically modified plants with higher yield and pathogenic resistance (Ceulemans et al. 1996, Anonymous 2006; see also Chapters 2 to 4 and 7 of this book). The biomass obtained from e.g. fast growing willow clones from plantations in western and central New York was estimated 10–13 dmt/ha/a (Heller et al. 2003).

Among the grasses, *Panicum virgatum* (extensively studied in the USA) and *Miscanthus* (studied in the EU) are the most promising plants. There are no studies available comparing systematically and directly both plants within one experiment. Nevertheless, the results obtained in the last years from individual studies indicate that *Miscanthus* \times *giganteus* produces about two times more biomass per area than *P. virgatum* (Heaton et al. 2004). Yields of *M. x giganteus* are more strongly dependent on water but *P. virgatum* is stronger controlled by N-availability. However, before choosing of a specific crop, parameters such as local climate and soil quality need to be considered (Lewandowski & Schmidt 2006, Tuck et al. 2006). Both grasses have a high potential for genetic improvements. *M. x giganteus* is a naturally occurring sterile hybrid with limited genetic variation (Jones & Walsh 2001) that still overcomes the biomass yield of improved *P. virgatum* varieties (Vogel & Masters 1998). Comprehensive energy balances have been made only very recently. The study of Acaroğlu & Askoy (2005) shows that the energy input which is needed for the cultivation of *Miscanthus* with the goal of high yields per hectare amounts to more than one third of the energy which is finally produced by the grass. This energy demand exceeds even the energy input which is needed to grow high yield agricultural crops like maize. Similar results have been obtained by Angelini et al. (2005) with giant reed (*Arundo donax*) in Southern Europe. These authors report 131 GJ/ha/a as highest energy yield which was possible without fertilisation, this value is equivalent to a timber yield of 9 tons. In another recent study, a best net energy yield of 590 GJ/ha/a was reported for *Miscanthus* under nitrogen supply (70 kg $\text{NH}_4\text{NO}_3/\text{ha/a}$) (Lewandowski & Schmidt 2006).

From Fig. 1 it is rather obvious that biomass is by far the cheapest way of producing energy as a renewable resource. The essential question is which type of biomass has the greatest economical advantages: trees or annual plants. Comparison of energy yields of different annual crop plants from the temperate zones with tree biomass (Fig. 3) leaves no doubt on the advantages of afforestation. The main reason for the better performance of fast growing tree species is the fast growth of biomass per hectare and year. Trees consist mainly of dead cells which contain no cytoplasm. In consequence, they need much less minerals and especially nitrogen than annual plants consisting of mainly living cells (trees 0.3% nitrogen compared to 4 to 7% in case of annual plants; Scheffer & Schachtschabel 1989).

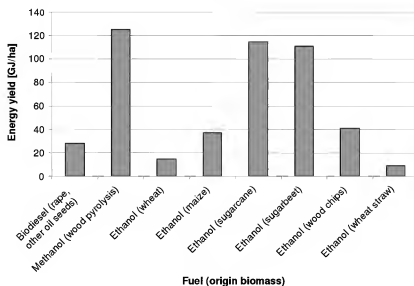


Fig. 3 Energy per area that can be produced by growing crop plants and timber (adopted from Sims et al. 2006)

Furthermore, trees have a much higher leaf area index. Thus, they catch much more light energy than annual plants. Calculated for the USA, 8,115 Mcal (34 GJ) of energy have to be invested for the production of 8.7 t of corn with a total energy content of 31,158 Mcal (130 GJ), presenting an input/output relation of 1 to 3.85 (Pimentel & Patzek 2005; Fig. 4). The main energy input in the produc-

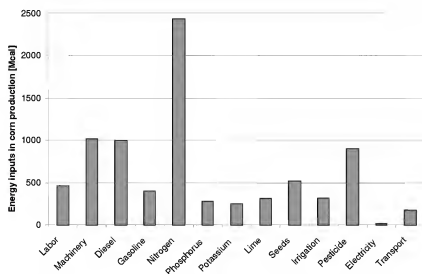


Fig. 4 Energy inputs necessary for growing annual plants: corn production of 8.7 t/ha in the USA (Pimentel & Platzek 2005); 1 Mcal = 4.18 MJ

tion of annual plants is caused by nitrogen fertilisation (Fig. 4) unlike in wood production that requires no or only low nitrogen fertilisation. Also, the required input of gasoline for the planting and harvesting machinery is much lower in wood production because of the comparatively long rotation times.

Processes for conversion of biomass into energy and chemicals

A number of different thermo-chemical processes can be used for conversion of biomass into energy (Fig. 5). The most important options are combustion, gasification, pyrolysis, and liquefaction (for overviews see McKendry 2002b, Bridgwater 2003, Bridgwater 2006). Selection of the technique depends mostly on type of biomass and the desired form of final energy (Kamm & Kamm 2007). Other aspects such as economic conditions (investment, process efficiency), biomass availability, and environmental concerns must also be considered.

Combustion

Burning of biomass is widely used to convert the stored chemical energy into heat for production of mechanical power and electricity. Combustion plants are built on different scales, from local power/heat units to large scale industrial plants with energy production of few thousand MW. Economically meaningful is combustion of dry biomass with moisture content below 50%. The overall energy conversion efficiency of this process is typically 20% for small combustion units and up to 40% for industrial plants (McKendry 2002b). Without further conversion

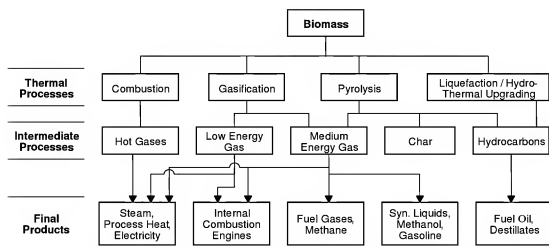


Fig. 5 Main thermo-chemical processes for biomass conversion and their final products (according to McKendry 2002b)

into mechanical and electrical power, combustion heat from furnaces can directly be applied to process cement, lime, tiles, and ceramics; the German cement plants need about 366,000 t/a of dry wood biomass that have to be produced yearly by afforestation (Marton & Alwast 2002; see also Chapter 5 of this book).

Gasification

Partial oxidation of biomass at high temperature, typically 800–900°C, converts biomass into a combustible gas mixture of carbon monoxide, carbon dioxide, hydrogen and methane. Two main types of gasifiers, fixed bed and fluidised bed, are used for this gasification process (Bridgwater 2003, McKendry 2002c). Gas as a low energy product can be potentially stored and transported; however, by cost reasons a produced gas mixture is usually immediately consumed in near-by power gas engines and gas turbines.

Alternatively, the gaseous products of gasification can be transformed at high temperatures to synthesis gas consisting of carbon monoxide and hydrogen. Clean synthesis gas ($\text{CO} + n\text{H}_2$, where n is in the range of 1–3) can be used for the production of all basic commodities needed by the chemical industry as well as of CH_4 -, respective CHO -fuels like diesel, methanol and ethanol. Synthesis gas can further be used in various other technical processes such as in the reduction of ores to metals and the homogenous Shift-reaction ($\text{CO} + \text{H}_2\text{O} = \text{CO}_2 + \text{H}_2$) allows the conversion of synthesis gas to hydrogen (Christen & Vögtle 1992).

However, the most important use for synthesis gas is the Fischer-Tropsch process where the mixture of CO and H_2 is transformed into liquid products (Dry 2002). Depending on the catalysts, a large variety of liquids can be synthesised, e.g. methanol, ethanol, and bio-diesel (linear long chained hydrocarbons). These primary products can further serve as the basis for synthetic organic chemistry and production of plastics and various other commodities. All chemicals made by this process, especially gasoline or diesel for cars, have a much higher purity than crude oil derivatives (Steiger 2003).

About 6,000 PJ/a of synthesis gas is produced worldwide, corresponding to almost 2% of the present total worldwide energy consumption (Boerrigter & van der Drift 2005). At present, 53% of the worldwide production of synthesis gas (obtained mainly from fossil energy sources like coal, gas, and refinery residues) is consumed by the ammonia producing industry. Only 19% of synthesis gas is used for production of liquid carriers such as methanol and diesel. According to the EU regulations, 5.75% of fuels must be renewable by 2010. It is expected that this value will increase to 10% by 2020 and to 20–45% by the year 2040 and that by this time the renewable fuels will correspond to an average world production of synthesis gas of 50,000 PJ/a (van der Drift et al. 2005, Plass 2005). From the environmental point of view and for economical reasons, a large part of the required biomass should be provided by wood (Maniatis & Millich 1998, Dowaki et al.

2005, Duret et al. 2005, Dahmen et al. 2007), waste wood products, and contaminated waste wood (Varvaeke et al. 2006).

The technology of wood gasification for energy production reached already an industrial scale. The European largest plant for production of electricity and heat from forest biomass started 2006 in Vienna-Simmering (Austria) with a capacity that serves 48,000 private households (Anonymous 2007). Costs for plants for the production of synthesis gas and costs for the following Fischer-Tropsch synthesis depend rather on their capacity. A capacity increase by an order of magnitude reduces the specific investment costs by a factor of ~ 2 (Henrich & Dinjus 2003).

Pyrolysis

Pyrolysis is the thermal conversion of biomass in the absence of oxygen at the temperature range of 400–500°C. Depending on the technique used, biomass can be converted to liquid (bio-oil or bio-crude), solid (charcoal), and gas fractions (Yaman 2004).

Production of bio-oil by flash pyrolysis, one of the most recently introduced renewable energy processes (see Czernik & Bridgwater 2004), allows biomass conversion to energy with 80% efficiency (Plass & Reimelt 2007). Essential for the process are a finely milled biomass, a controlled reaction temperature, a short vapour residence time, and rapid cooling of the pyrolysis vapours (Bridgwater 2003). The readily stored and transported bio-oil can replace diesel and fuel oil in all kinds of engines, furnaces and turbines. Bio-oils by now have found applications in commercial production of chemicals and new bio-oils are being developed for replacing other fuels. In addition, bio-oils can be used as feedstock for the production of raw synthesis gas. A pilot plant utilising 17,000 t/a of straw and other agricultural waste materials started in 2007 in Freiberg (Germany) as a cooperation project of the Lurgi AG and the Forschungszentrum Karlsruhe (FZK; Hoffmann 2007).

Charcoal, the solid by-product of pyrolysis is also a valuable energy resource (Ryu et al. 2007). In a model project from the Prototype Carbon Fund of the World Bank (Plantar, Brazil), several plants are in operation for charcoal production which is used for the processing of ore to pig iron. For the supply of timber, 23,000 ha forests were planted with high yield clones of Eucalypt (Anonymous 2002).

Pyrolysis can be also applied to produce energy from waste wood and contaminated wood (Bridgwater et al. 1999). According to the Bavarian Environmental Protection office, disposal costs for wood wastes in Germany varied in 2002 from 13 to almost 230 €/t or even higher (in average 150 €/t) for wood contaminated with chlorinated compounds (Gruber et al. 2004). This kind of biomass – estimated as 7.9 million tones yearly in Germany (Müller-Langer & Thran 2006) – can be converted to liquid fuels leaving the inorganic preservatives (chromium, copper,

alumina, and boron salts) in the char fraction (Helsen et al. 1998, 2003, Mohan et al. 2007).

Hydro-thermal upgrading and liquefaction

Besides from pyrolysis, bio-oils can be also obtained from hydro-thermal upgrading of biomass at high pressure. The process is performed under wet conditions and can utilise e.g. wet biomass from agro-forestry. However, this type of biomass conversion is still in the developing and scaling-up stage and must be assessed under industrial commercial conditions in the future (McKendry 2002b).

Even less developed are liquefaction processes such as the treatment of biomass in low temperatures and at low hydrogen pressures and the treatment of biomass with water and catalyst at high temperatures (Zhong & Wei 2004). At the laboratory scale, both processes operate satisfactorily. However, at present they are regarded to be less economical than pyrolysis.

Fermentation of biomass

The oldest fermentation we know is the production of alcohol by anaerobic fermentation of glucose with yeast. It was developed by virtually all early civilisations more than 5,000 years ago (Piskur et al. 2006). This process in its contemporary degree of sophistication (Olsen & Schäfer 2006) is still today the dominating biotechnological process in the production of an individual chemical. In 1915, the process for the fermentation of starch to butanol and acetone was brought to industrial production by Chaim Weizman (see McCutchan & Hickey 1954). Since then, fermentation of biomass with specific micro-organisms as a production process has been developed for only a few more mass chemicals (Hermann & Patel 2007). All these processes have in common that the starting material is always an aqueous solution of pure sugars, mainly glucose (Vane 2005, Lin & Tanaka 2006), and that after fermentation the target chemical has to be separated from the water by distillation.

Different chemical and physical pretreatments can be applied to biomass in order to release soluble polysaccharides and to subsequently enhance their enzymatic degradation to simple sugars (Sun & Cheng 2002, Wyman et al. 2005a,b, Lin & Tanaka 2006) prior to the microbial ethanol fermentation. This type of aerobic fermentation can be applied for conversion of "wet" biomass into chemicals such as ethanol, butanol or acetone (Sheehan & Himmel 1999, Kempainen & Shonard 2005). Alternatively, anaerobic fermentation of biomass by a spontaneously generated micro-flora or selected micro-organisms is used to produce so called biogas consisting mostly of methane (Gunaseelan 1997). Pimentel and Patzek (2005) made an energy balance for the production of ethanol from either corn, switch grass, or wood cellulose. Table 2 gives the results for corn showing that the total energy input in ethanol production exceeds the energy out-put. For ethanol

Table 2 Inputs per 1,000 l of 99.5 % ethanol produced from corn (adopted from Pimentel & Patzek 2005)

Inputs	Quantity	kJ x 1000	Costs US \$
Corn grain ¹	2,690 kg	10,522	284.25
Corn transport	2,690 kg	1,347	21.40
Water	40,000 l	377	21.16
Stainless steel	3 kg	50	10.60
Steel	4 kg	50	10.60
Steam	10,642 MJ	10,652	21.16
Electricity	392 kWh	4,230	27.44
Conversion of 95% to 99.5% ethanol	9 Mcal	38	40.00
Sewage	20 kg BOD ²	289	6.00
Total		27,602	453.21
Energy of 1,000 l ethanol		21,464	

¹ Only those energy is considered which is required to grow and harvest the corn.

² Wastewater with a biological oxygen demand (BOD)

production from switch grass and from wood, the energy balance is also negative (Patzek & Pimentel 2005, Pimentel & Patzek 2005).

Bio-diesel from plant seeds

Various seeds of annual plants can be used to produce vegetable oils and, after further chemical conversion, to replace diesel. The best tested and applied candidate plants for this technology are sunflower, soybean and rape. However, if the target for biomass production is to obtain as much net energy per land area as reasonably possible, these crops perform rather poorly. For all three plants, the processes of vegetative oil production and chemical conversion to bio-diesel need more input of energy in form of fossil fuels than what the final product contains (rape: Friedrich et al. 1993; soy beans and sunflowers: Pimentel & Patzek 2005). Table 3 presents soybeans as an example of how cost calculations are done and which factors are considered.

Soybeans contain less oil than sunflower: 18% soy oil and 26% sunflower oil can be obtained from the same amount of biomass. However, soybean can be produced without (or almost without) nitrogen supplementation thus saving high costs of nitrogen fertilisation. Nevertheless, the energy return in the conversion of soybean into biodiesel is strongly negative: as calculated by Pimentel and Patzek (2005), 1 energy unit of biodiesel from production from soybean requires an input of 1.24 units of primary energy, at present fossil fuels. In the same publication, the authors report a negative input/output energy ratio for sunflower of 1 to 1.18.

Table 3 Inputs per 1,000 kg of biodiesel oil produced from soybeans (adopted from Pimentel & Patzek 2005)

Inputs	Quantity	kJ x 1000	Costs US\$
Soybeans	5,556 kg	32,635	1,117.42
Electricity	270 kWh	2,916	18.90
Steam	5,643 MJ	5,648	11.06
Cleanup water	669 MJ	669	1.31
Space heat	635 MJ	632	1.24
Direct heat	1,839 MJ	1,841	3.61
Losses	1,254 MJ	1,255	2.46
Stainless steel	11 kg	661	18.72
Steel	21 kg	1,029	18.72
Cement	56 kg	444	18.72
Total		49,698	1,212.16
Energy value of 1,000 kg of biodiesel		37,656	

Perspectives of the renewable energy projects

The above data clearly indicate that both the extraction of oil from oil seeds and the conversion of biomass into ethanol – even in the form of almost 100 % pure starch or cellulose – have a negative energy balance. The input which is required for the production of the energy carriers presents a higher energy content than the product finally has. In addition, there are socio-economical concerns. For example, expanding ethanol production might cause a conversion of valuable cropland from producing corn needed for food to producing corn for ethanol factories. The success of running projects seems to be related rather to political reasons (agricultural subventions) than to true economic cost calculations (Patzek 2004).

Afforestation and the use of timber for the substitution of fossil fuel as an energy source is at present the only feasible way for the transition of the global economy to sustainability. The White Paper of the EU (Anonymous 1997) opens an attractive possibility for the participation of afforestation projects in the CO₂-certificate trading process. Replacement of power and heat production from conventional fossil fuels with renewable energy sources is the cheapest option to reduce greenhouse gas emissions from power and heat production from the atmosphere (for more details see Chapter 5 in this book). Pollution by SO₂ and NO_x gases can be reduced by using woody biomass to levels comparable with photovoltaic and wind energy (Demirbaş 2004, Heller et al. 2004).

Energy from biomass, preferentially from wood biomass, has already a broad acceptance in many countries. Developing countries with high populations and industrialisation potential such as Brazil, China and India are strongly interested in biomass usage. About 24% (Sudha et al. 2003) and more than 8% (Chang et al.

2003, Junfeng & Runqing 2003) of energy demand projected for the year 2010 in India and China, respectively, could theoretically be covered by fuels produced from wood. Both countries have large areas available for afforestation and costs calculations proved very good economic benefits. However, especially for the developing countries it may be hard to come from the theoretical benefits to the practice. High initial investment, long payback period, technical and logistic concerns and social aspects of present agriculture systems are at present very difficult to overcome. Problems of establishing effective technologies for biomass conversion in Europe are of different nature. The most powerful promoting force for renewable energy is the public opinion and acceptance. However, public distrust towards new, unfamiliar technology and lack of understanding of economical and ecological advantages by diverse local groups in regions of implementation can result in a very serious barrier in implementation of RES (Upreti 2004). Proper information policy gives the up-coming bioenergy technology the best possible promotion.

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7. Transgenic Trees

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Introduction

The transfer of genes into bacteria, fungi, plants or animals results in genetically modified or transgenic organisms. This technique allows to cross the species boundaries by introduction of foreign genes into a host genome and permits to control the expression strength and expression pattern of the transgene by selection of a suitable promoter. Transgenic organisms are well established tools in basic research and allow to study the function of genes by overexpression and knock outs. In applied sciences, the production of transgenics aims at producing organism with new or superior characteristics. In tree breeding, genetic engineering has a high application potential since it accelerates the introduction of new traits into a given genetic background by circumvention of the long generation

time of trees, which need years or even decades from germination to flowering (Merkle & Dean 2000). Some application areas of genetic engineering of trees are the same as in other economic plants, such as pest control and herbicide resistance (for examples, see Table 1). We will briefly address these topics but focus on tree specific properties like modification of lignin content or structure and wood quality and wood production (see also Table 1). Finally, the risks and potentials of transgenic trees will be discussed as well as aspects which specifically have to be considered with respect to the production of transgenic trees.

Transformation techniques

The principle steps of transformation are the introduction of a novel gene into the genome of a host plant and the regeneration of rooted plantlets from single, transformed cells. Therefore, an important prerequisite for the production of transgenics is the availability of tissues that can be transformed and which have a high regenerative potential. Examples for tissues of trees with these features are leaf and stem explants, and cultures of somatic embryos (Fladung et al. 1997, Tzfira et al. 1997, Ismail et al. 2004). Organogenesis from leaf and stem explants usually proceeds via a callus stage which forms micro-shoots that can be rooted. Somatic embryos are derived from somatic plant cells instead from gametes (Finer 1994, Pena & Séguin 2001). They contain root and shoot axes and have the capacity to germinate and form a complete plant.

Nowadays, three general methods are widely established for the introduction of genes into plants: *Agrobacterium* mediated transformation, the biolistic method using a particle gun, and transfer of DNA by electroporation or polyethylene glycol into protoplasts (Tang & Newton 2003). The use of *Agrobacterium* has several advantages over the other methods, e.g. integration of a limited number of transgene copies into the host genome and stability of the transferred DNA. However, not all plants are amenable to this transformation vector. Angiosperm trees like *Populus* and *Eucalyptus* are routinely transformed with *Agrobacterium*, while conifers have proven to be more recalcitrant to this transformation method. Successful transformation of conifers has been achieved with the biolistic method. But also attempts are underway to use hypervirulent *Agrobacterium* strains for gene transfer to conifers.

The soil bacterium *Agrobacterium* is a “natural genetic engineer” because it inserts part of its own DNA into the nuclear plant genome, thereby redirecting the programming of the cell to proliferation and formation of substrates for its own growth (Wood et al. 2001). The transfer of the DNA sector that is confined by left and right border from the tumor-inducing (Ti) plasmid occurs with the help of virulence (*vir*) genes (Fig. 1). A transgene that has been inserted between left and right border, thereby replacing the *Agrobacterium* genes and recombining transgene DNA and *Agrobacterium* DNA, will be transferred into a host plant in

Table 1 Transgenic trees in applied research

Engineered trait	Species	Gene	Reference
Lignin content and structure	<i>Populus spec.</i>	4Cl antisense	Hu et al. 1999
	<i>Populus spec.</i>	F5H overexpression	Franke et al. 2000
	<i>Populus spec.</i>	CCoAOMT antisense	Meyermanns et al. 2000
	<i>Populus spec.</i>	COMT antisense	Tsai et al. 1998, Jouanin et al. 2000
	<i>Populus spec.</i>	CAD antisense	Baucher et al. 1996, Lapiere et al. 1999
	<i>Populus spec.</i>	F5H overexpression and 4Cl antisense	Li et al. 2003
Lignin and cellulose content, fibre length	<i>Populus spec.</i>	GA 20-oxidase	Eriksson et al. 2000
Resistance to abiotic stress	<i>Liriodendron tulipifera</i>	mercury reductase	Rugh et al. 1998
	<i>Populus spec.</i>	γ -ECS	Noctor et al. 1996, Peuke & Rennenberg 2005
	<i>Populus spec.</i>	glutathione reductase	Foyer et al. 1995
	<i>Populus spec.</i>	GS	Strohm et al. 1995, 1998, 2002
Fungal resistance	<i>Malus domestica</i>	antifungal chitinase	Bolar et al. 2000
	<i>Populus spec.</i>	chitin binding peptide	Liang et al. 2002
Insect resistance	<i>Populus spec.</i>	proteinase inhibitor	Delledone et al. 2001
	<i>Populus spec.</i>	Bt	Hu et al. 2001, Kleiner et al. 2003
Virus resistance	<i>Carica papaya</i>	viral coat protein	McLean & Charest 2000
Herbicide resistance	<i>Picea abies</i> ,	<i>bar</i>	Ellis et al. 1993
	<i>Pinus radiata</i>		
	<i>Populus spec.</i>	EPSP synthase	Fillatti et al. 1987
	<i>Larix</i>	EPSP synthase	Shin et al. 1994
Flowering	<i>Citrus sinensis</i>	LFY, AP1	Pena et al. 2001

the process of the infection with the recombinant *Agrobacterium* strain (Fig. 1). The infection and DNA transfer involve several steps which include binding of *Agrobacterium* to a plant receptor and sensing of phenols secreted by the plant that induce the transcription of the *vir* genes (Sheng & Citovsky 1996). The fact that the host plant has to be recognised results in host specificity of *Agrobacterium*. Therefore, only a limited number of plant species can be transformed by *Agrobacterium*.

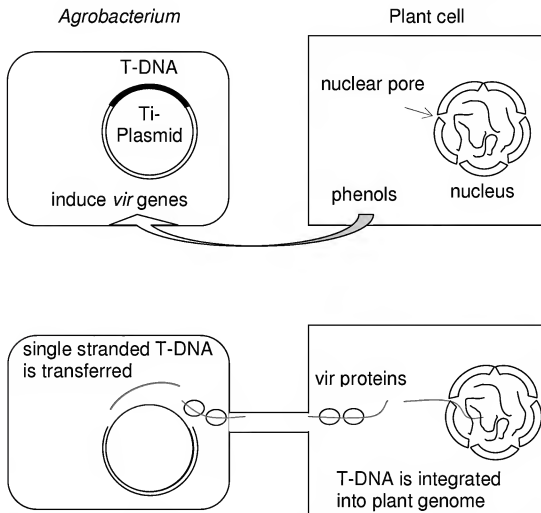


Fig. 1 *Agrobacterium* mediated transfer of genes into a plant genome (adapted from Heldt 1997)

In that respect, the biolistic method is more robust and can be applied for gene delivery to any plant species and plant tissue (Birch 1997). The device for biolistic introduction of DNA into tissue was, appropriately enough, named “gene gun”. The transgene DNA is bound to tungsten or gold particles and then “shot” by pressure into the target tissue. The disadvantage of this method is that quite often a high number of transgenes is integrated into the host genome. This increases the probability of gene silencing, a phenomenon that leads to the unwanted effect that the integrated transgene shows variable expression patterns or even is not expressed. However, stable transformations of several conifer species have been achieved with this method (Tang & Newton 2003).

The third method used for gene transfer is limited to DNA transfer into protoplasts. Electroporation induces transient openings in the plasma membrane

(Birch 1997) and allows the transgene DNA to enter the cell. Alternatively, polyethylene glycol (PEG) has been used which binds the transgene DNA and is taken up into the cell by endocytosis as a DNA-PEG complex. The transformation of protoplast has been applied for the transient (temporary) expression of transgenes to evaluate promoter activity and functionality of transgene constructs (Bekkaoui et al. 1988, 1990, Tautorus et al. 1989). The activity of promoters connected to a reporter gene encoding for example β -glucuronidase or green fluorescent protein can be rapidly assessed under the microscope.

The first transgenic tree produced was a poplar (Fillatti et al. 1987). The gene *araA* encoding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) was transferred by *Agrobacterium*-mediated transformation and provided resistance to glyphosate herbicides, which are used in weed control (see below). The availability of transformation protocols for some poplar species and the sequencing of the genome of *P. trichocarpa* (Tuskan et al. 2004) made poplar the angiosperm model tree. Huang et al. (1991) achieved the stable transformation of a conifer using *Agrobacterium*. Today transformation of many deciduous trees and conifers is well established. Transformation protocols not only exist for model trees but also for several commercially important species of the genus *Populus*, *Eucalyptus*, *Malus*, *Pyrus*, *Citrus*, *Picea*, *Pinus*, *Larix* (Ellis et al. 1993, Shin et al. 1994, Tzfira et al. 1997, Bell et al. 1999, Pena et al. 2001, Szankowski et al. 2003, Tournier et al. 2003).

Strategies to change the gene expression level

The aim of many genetic engineering approaches is to increase, decrease or completely turn off the activity of a gene. Some applications direct the expression to a specific target tissue or target cells. The expression strength and tissue specificity is determined by the promoter of a gene. Recombinant DNA technology allows to combine any promoter with a gene of interest to achieve constitutive, inducible or tissue specific expression. A widely used promoter is the 35S promoter from the cauliflower mosaic virus which provides strong and constitutive expression in almost all plant tissues. In poplar, the 35S promoter has a high activity in all tissues except for the cambium and xylem, and it shows no seasonal dependency (Nilsson et al. 1996a). An alternative to the 35S promoter is the synthetic promoter Emu (Merkle & Dean 2000). A loss or decrease in the activity of a gene can be achieved by either knock-out, antisense constructs or RNAi (McGinnis et al. 2005). Both of the latter techniques exploit a general defence strategy of plants against viral RNA that detects and degrades double stranded RNA. A complete knock out of a gene is possible by using T-DNA constructs carrying only a selectable marker (Alonso et al. 2003) or transposons (Wisman et al. 1998). In both cases, the insertion of a DNA fragment physically interrupts a gene which in most cases leads to loss in gene function. Promoter trap approaches led to the identification of tissue specific

formation of an ether bond but prevents a carbon-carbon bond at this position. The different types of bonds have severe impact on the delignification process during pulp and paper making (Chen et al. 2001). Lignin has to be removed from the pulp since it negatively influences the brightness of the produced paper. The chemical delignification by Kraft pulping cleaves the ether bonds but leaves the carbon-carbon bonds intact. Therefore, the lignin from gymnosperms is more difficult to extract due to the high amount of carbon-carbon bonds formed between the monomeric subunits while many chemical bonds in the angiosperm lignin are of the ether type that allow an efficient delignification. The characteristics of angiosperm lignin are determined by the ratio of guaiacyl to syringyl residues (S/G ratio).

The goal of genetic engineering with respect to pulp and paper making is to generate trees that produce wood with a lower lignin content and to obtain genetically modify angiosperm trees that contain lignin with a higher proportion of sinapyl to guaiacyl residues. The targets of genetic engineering are the genes that encode the enzymes of monolignol biosynthesis. The starting point of this biosynthetic pathway is phenylalanine (Fig. 2). Phenylalanine ammonium lyase deaminates phenylalanine to cinnamic acid, which is hydroxylated by cinnamate 4-hydroxylase (C4H) to *p*-coumaric acid. 4-coumarate:coenzyme A ligase (4CL) activates *p*-coumaric acid by forming a thioester bond with coenzyme A. The thioesters are reduced to aldehydes and these are reduced to the monolignols by cinnamyl alcohol dehydrogenase (CAD). Ferulic acid 5-hydroxylase (F5H) introduces a hydroxyl group at the aromatic ring. Caffeic acid-O-methyl transferase (COMT) and caffeoyl-coenzyme A-O-methyltransferase (CCoAOMT) methylate hydroxyl groups. Both reactions are important for the introduction of methoxy groups at the aromatic ring which determine the extractability of lignin (see above). Significant changes of lignin content and S/G ratio in trees have been achieved by genetic engineering of *4CL* (Hu et al. 1999), *F5H* (Franke et al. 2000), *CCoAOMT* (Meyermanns et al. 2000), *COMT* (Tsai et al. 1998, Jouanin et al. 2000) and *CAD* (Baucher et al. 1996, Lapierre et al. 1999).

Transgenic poplars expressing *4CL* in antisense exhibited a 90% decrease in 4CL activity. Decreasing 4CL activity caused a 45% reduction in lignin content with no change in the ratio of guaiacyl to sinapyl residues (Hu et al. 1999). The significant reduction in lignin had the additional effect that the cellulose content of the wood was increased by 15% and the transgenics exhibited higher growth rates both in height and stem diameter. Another attempt using transgenic poplars aimed at an increase of the syringyl to guaiacyl ratio in lignin (Franke et al. 2000). The overexpression of F5H under the control of the *C4H* promoter, which allows expression of F5H in xylem parenchyma, increased the methoxylation of the monolignol residues. The wood of F5H overexpressing poplars had lignin with more syringyl units than the control plants (Franke et al. 2000). It is expected that

this change allows a better extractability of lignin, but the quality of pulp from trees overexpressing F5H has not been tested yet.

Downregulation of *CCoAOMT* yielded poplars that had a 12% reduction in lignin and unexpectedly an increase in the S/G ratio (Meyermanns et al. 2000). Processing of the wood gave a higher pulp yield with lower lignin content in the pulp (Chen et al. 2001). Poplars carrying the antisense *COMT* gene revealed that the effect of genetic engineering can vary significantly depending on the degree of downregulation of the target gene (Chen et al. 2001). A 97% reduction of *COMT* resulted in a 17% reduction in lignin, a 6% higher cellulose content and a 10% increase in pulp yield. A negative effect of the *COMT* downregulation was, as expected, a decreased S/G ratio, which caused a higher kappa number compared to controls indicating a higher lignin content of the pulp. Smaller reductions of *COMT* activity had no effect on the lignin content (Van Doorselaere et al. 1995, Tsai et al. 1998, Chen et al. 2001).

A natural pine mutant of *CAD* as well as antisense *CAD* poplars are famous for their red xylem which is caused by the higher content of cinnamyl aldehydes in the lignin (see cover picture of this book). These trees had a reduction in lignin content of 5% and yielded pulp with a lower kappa. The higher lignin extractability was not caused by a change in the S/G ratio but instead by a high content of free phenolic groups (Lapierre et al. 1999).

More advanced techniques for modification of lignin content and composition aim at changing the expression of two or more genes simultaneously. This has been reported by Li et al. (2003), who produced a poplar line overexpressing F5H with a reduction in 4CL by using *Agrobacterium* co-transformation. The resulting poplar line combined a 54% decrease in lignin content with an increase in the S/G ratio. These results show that it is possible to genetically engineer trees in a way that they produce wood that will need less chemicals for pulping and bleaching.

Lignin content is only one aspect of wood quality. With respect to construction wood, furniture, fibre boards, etc., wood properties like wood density, fibre length, cell wall characteristics of fibres and tracheary elements, growth ring width, proportion of compression wood and lateral branching are more important than lignin content and structure (Savidge 2003). The wood industry considers wood density as an important and easy to measure parameter of wood quality, since wood density is correlated to wood strength (Rozenberg & Cahalan 1997, Savidge 2003). In angiosperm trees, the wall thickness of xylem cells and the ratio of fibres to vessels determine wood density. Thick cell walls and a high proportion of fibres are characteristics of wood with high density values. Since late wood cells have smaller cell lumina and thicker cell walls than early wood cells, a high proportion of late wood has a positive effect on wood density in angiosperm and gymnosperm trees.

The mechanical strength of the individual wood cell is another factor that has been thoroughly investigated with respect to wood quality. This parameter is clearly influenced by the angle of the cellulose microfibrils in the S2 layer of the secondary cell wall. This angle is defined by the predominant orientation of the microfibrils in relation to the long axis of the cell. A high cell wall stiffness is achieved by low microfibril angles between 0 and 15° (Cave 1968). These examples exemplify that wood quality is determined by complex traits which are not likely to be determined by one gene. Moreover, genetic engineering of wood quality has been hampered by the fact that not enough information is available on the biological bases that modulate and control vessel and fibre development.

Hormonal control of xylem differentiation

Many investigations of wood development addressed the role of plant hormones in the activity and differentiation of the vascular cambium. A focus has been the effect of auxin and auxin transport inhibitors (Aloni & Zimmermann 1983, Zakrewski 1991, Sundberg 2000, Teichmann 2001, Junghans et al. 2004). Other hormones known to influence cambial activity and differentiation in interaction with auxin are cytokinin and gibberellin (Wareing 1958, Kijidani et al. 2001). First attempts to change the hormone contents in trees by transfer of genes involved in hormone biosynthesis and physiology had a purely scientific basis with the goal to study the effect of increased cytokinin and auxin contents on tree development (von Schwartzberg et al. 1994, Tuominen et al. 1995, Nilsson et al. 1996b, Grünwald et al. 2000).

The transfer of the cytokinin biosynthesis gene *IPT* into poplar had severe effects on tree phenotype. The plants exhibited reduced apical dominance and were unable to root (von Schwartzberg et al. 1994). Expression of *IPT* under the control of the auxin sensitive GH3 promoter resulted in poplar plants that did not form roots and had a bushy phenotype (Bolu, unpublished data, Fig. 3). Transformation of poplar with the *rolC* gene from *Agrobacterium rhizogenes* caused stunted growth with an increased number of small leaves (Nilsson et al. 1996b). The function of the *rolC* gene has not been elucidated yet, but it seems to be involved in cytokinin activity or metabolism. The *rolC* transgenic poplars had lower auxin and gibberellin contents, while the biosynthesis of the cytokinin conjugate zeatin 9-riboside was increased. Analysis of the wood of *rolC* poplars revealed atypical late wood formation with thin-walled fibres (Grünwald et al. 2000). Introduction of the *Agrobacterium* auxin biosynthesis genes *iaaM* and *iaaH* into poplar caused an increase of auxin in leaves and roots. The growth rate of the transgenic trees was significantly reduced and the wood anatomy showed changes with reduced vessel size, increased vessel density and fewer rays (Tuominen et al. 1995). These investigations showed that changes in hormone balance and content can be achieved in trees by molecular methods and the results are important for the basic under-



Fig. 3 Phenotype of transgenic poplar expressing the cytokinin biosynthesis gene *IPT* under the control of the *GH3* promoter

standing of hormone function in trees, but the produced transgenics with changes in the auxin and cytokinin content did not have the potential for commercial use.

However, investigations on the effect of increased gibberellin contents in poplar raised hopes that manipulation of this class of phytohormones may allow to improve tree growth, biomass production and fibre characteristics. Hormone application studies showed that gibberellins are important for xylem fibre differentiation and influence fibre length (Wareing et al. 1958, Digby & Wareing 1966, Ridoutt et al. 1996). The overexpression of a key enzyme of the gibberellin biosynthetic pathway (GA 20-oxidase) under control of the strong, constitutive 35S promoter increased the gibberellin content in leaf and stem tissue of transgenic poplar and resulted in faster growth with increased biomass production (Eriksson et al. 2000). The xylem of the transgenics contained fibres with an 8% increase in length compared to non-transformed wildtype poplars. A drawback of the genetic manipulation was a poor root initiation of the transgenic poplars which caused problems during the transfer of regenerated plantlets into soil.

Taken together, the results show that manipulation of hormone content of poplar can lead to trees with superior characteristics, but exemplifies that it may be important to confine the expression of the transgene to the target tissue by using tissue-specific promoters. This will avoid negative effects of the overexpression in non-target tissues.

Tolerance to abiotic stress

In plants, a change in environmental conditions that negatively influences plant growth or development is considered as stress (Levitt 1972). There are many abiotic factors which may cause stress, like extreme temperatures, high or low light and adverse soil conditions (e.g. high content of heavy metals or salt, unfavourable pH, poor nutrient availability). Each of these stress factors has specific effects in the plant which are typical of the stressor, but there are also common signaling pathways and response patterns to many stress conditions (Seki et al. 2003, Polle et al. 2006).

Many stressful conditions cause formation of reactive oxygen species. The formation of superoxide (O_2^-), hydroxyl radicals (OH^\cdot) and singlet oxygen (1O_2) during aerobic metabolism is caused by electrons which leak from electron transport chains in chloroplasts and mitochondria. This process is aggravated by many stress forms and induces a radical-scavenging, antioxidative system (Polle 1996, 1997, 1998, Polle et al. 2000, Polle and Schützendübel 2003, Gratao et al. 2005).

The tripeptide glutathione (GSH) detoxifies reactive oxygen species directly and is also involved in the regeneration of the radical scavenger ascorbate in the ascorbate-GSH cycle (Polle 1996). The function of GSH is due to the reversible oxidation of the SH group of the amino acid cysteine. GSH molecules that have been oxidised during the detoxification process to GSSG can be regenerated through reduction by glutathione reductase (GR). The biosynthesis of GSH occurs in two steps. Cysteine reacts with glutamate in a reaction that is catalysed by γ -glutamylcysteine synthetase (γ -ECS). GSH synthetase (GS) adds a glycine to yield GSH. The fact that many stress forms cause the production of reactive oxygen species make enzymes or components of the antioxidative system interesting targets for the engineering of stress resistance. Therefore, poplar has been transformed with the bacterial gene for γ -ECS or that for GS to increase the GSH content (Strom et al. 1995, Arisi et al. 1997, Noctor et al. 1996). The overexpression of GS has neither an effect on the GS content nor on stress tolerance (Strohm et al. 1995, 1998, 2002). The overexpression of γ -ECS resulted in increased GSH levels in leaves and roots of transformed poplars but unexpectedly did not change the resistance of the transgenics to experimentally induced stress by the herbicide paraquat, which injures plants due to increased superoxide radical production. Overexpression of GR targeted to the chloroplast increased GSH content and reduction state in leaves but again had no effect on the resistance of

the transgenic poplars to paraquat. However, the GR transgenics were slightly less sensitive to photoinhibition by high light than wildtype plants (Foyer et al. 1995). Tolerance for ozone, an oxidising air pollutant, was not increased (Strohm et al. 2002).

Studies on the overexpression of genes involved in GSH biosynthesis and regeneration did not result in poplar plants with significantly increased stress resistance but paved the way to a different but similarly important application. The metal chelating peptide phytochelatin (PC) consists of repeated γ -glutamyl cysteine subunits and is synthesised from GSH by phytochelatin synthase (Schützendübel & Polle 2002). PC binds heavy metals and the resulting complex is detoxified by transport to the vacuole. GSH over-expressing poplars had a higher capacity to increase PC content upon exposure to cadmium than wildtype plants. The GSH transgenics did not show a higher tolerance to the toxic heavy metal but accumulated more Cd than non-transgenic poplars. This phenomenon can be exploited in phytoremediation, a technique to remove contaminating substances like heavy metals or organic compounds from soil (Pilon-Smits 2005). Plants that have a high capacity to take up contaminants are planted on the polluted area. Harvest of the plants removes the accumulated contaminant from the area and leads to improvement of soil quality. The plant material is combusted and then deposited on a waste site. Trees used in phytoremediation offer some advantages over herbaceous plants. Leaves with the accumulated contaminants can be repeatedly removed from the side each autumn without the need of a replantation for several years. After the final harvest, wood from the trees can be used for energy production in special power plants. A field trial with poplars over-expressing γ -ECS on copper contaminated field sites has been started in Germany and in parallel in Russia (Peuke & Rennenberg 2005).

Another phytoremediation approach was reported for transgenic yellow poplar (*Liriodendron tulipifera*) over-expressing the bacterial gene for mercuric reductase. These plants were resistant to toxic levels of mercuric ions (Rugh et al. 1998). A drawback of the approach was the release of elemental mercury by the transgenics which posed a direct danger to human and animal health and made these trees unsuitable for application in phytoremediation.

Since salinity is a world-wide problem, there have also been various approaches to improve the salt tolerance of plants. While most projects focused on improving salt tolerance in agricultural crops, recently salt tolerance in tree species has also been addressed. Hu et al. (2005) increased the mannitol concentration of poplar by overexpression of the *mtlD* gene and showed that the transgenic poplars were more salt-tolerant than the wildtype. So far, no transgenic trees with increased drought tolerance are available (Polle et al. 2006). This is surprising, because drought is globally one of the most limiting factors for plant growth and productivity.

Biotic stress

Plants are attacked by an armada of biotic enemies, e.g. herbivorous animals, insects and nematodes. Fungi, bacteria and viruses cause a multitude of plant diseases. A frequently used technique in pest management for prevention or control of insect infestation is the application of the *Bacillus thuringiensis* toxin (Bt). This protein is built in the bacterium *Bacillus thuringiensis* as a non-poisonous protoxin and becomes activated in the intestine of certain insects. There, it binds to specific receptors and, by harming the intestinal wall, kills the insect (further reading in Chapter 14 of this book). Bt is presumably non-toxic for humans. Therefore, Bt compounds that are applied by spraying are approved in organic farming. The bacterial gene *Cry1a* encoding the Bt toxin has been introduced in maize and cotton. These crop plants produce the bacterial protein and are resistant to insects like the European corn borer (*Ostrinia nubilalis*) and the scarce bordered straw (*Helicoverpa armigera*). Transgenic poplar plants expressing the Bt protein were protected against the larvae of the gypsy moth and forest tent caterpillar (Kleiner et al. 2003). A field trial in the Xinjiang Uygur Autonomous region in China has proven the protective effect of Bt in trees under field conditions (Hu et al. 2001; further details in Chapter 14 of this book). The Bt gene has been introduced into several other tree species like walnut and white spruce (Ellis et al. 1993, Dandekar et al. 1998). As a different approach to engineer insect resistance, proteinase inhibitors have been expressed in poplar (Lep   et al. 1992, Klopfenstein et al. 1997). Increased resistance against the fungal pathogen *Septoria musiva* has been achieved in poplar by expression of chitin binding peptides (Liang et al. 2002). The strategy to protect plants against viruses by expressing the virus coat protein was applied in papaya trees (Chiang et al. 2001). The papaya ringspot virus destroyed 40% of the papaya trees on Hawaii and threatened the \$ 14 million-a year papaya industry. In 1998, seeds engineered for virus resistance were the first and up to now only commercially released transgenic trees in the USA and saved the papaya production on Hawaii (McLean & Charest 2000).

Herbicide resistance

The herbicides glyphosate and glufosinate (phosphinotricin) are toxic to almost all plants, but have the advantage of biodegradability. They could not be applied to crops before the development of plants genetically engineered for herbicide resistance. Glyphosate is structurally similar to phosphoenol pyruvate. It interrupts the shikimate pathway by competitive binding to the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), which shuts off the production of aromatic amino acids. Plants overexpressing a bacterial EPSP, which is less sensitive to glyphosate, are protected against this herbicide. Glufosinate as a structural analogon of glutamate inhibits glutamine synthase. Plants treated with glufosinate accumulate toxic amounts of ammonium and die. Resistance to glufosinate was achieved

by introducing the *bar* gene from *Streptomyces hygroscopicus* encoding phosphonotri-cine acetyl transferase, an enzyme that detoxifies this herbicide (Ellis et al. 1993).

Herbicide resistance is commercially very attractive for companies since glyphosate and glufosinate are sold as so called complementary herbicides, which can only be applied to genetically engineered plants offered by the same company. With respect to trees, glyphosate and glufosinate may be applied to control weeds during the establishment and maintenance of tree plantations (Walter et al. 2002). The first transgenic tree was a poplar engineered for lower glyphosate susceptibility by overexpression of the EPSP synthase (Fillatti et al. 1987). The same approach was chosen for European larch (Shin et al. 1994). Glufosinate resistance was reported for *Picea abies* and *Pinus radiata* transformed with the *bar* gene from *S. hygroscopicus* (Bishop-Hurley 2001).

Risks and Potentials

Genetic engineering is not a new technique. The first recombinant DNA molecule was produced in 1972 in the laboratory of Paul Berg (published in Jackson et al. 1972). In 1975, safety measures for the use of genetically modified organisms were defined at the Asilomar conference. In 1982, Schell et al. reported on the transformation of plant cells. But admittedly, the use of genetic engineering has not the same "established history of environmental safety" as conventional plant breeding (McLean & Charest 2000). Therefore, risk assessment studies have to be carried out. Two aspects are important in this context: the studies have to be done case-by-case, since many investigations have shown that a gene may behave differently depending on the integration site and the genomic background of the transformed plant. Secondly, release experiments are mandatory, since the expression rate and stability of transgenes may differ between green house and field conditions. Moreover, the realistic monitoring of the effect of transgenics on its symbionts and pathogens is only possible in a natural environment. Field experiments also allow to determine the rate of horizontal gene transfer, that is the transfer of genes between kingdoms. Release experiments in USA, Canada and Germany have shown that it is possible to define regulations that allow to carry out these experiments with assessable risk for the environment. Compared to crops, genetically modified trees are more challenging in risk assessment studies. Their long life span makes studies on transgene stability in trees a priority but this may be very time consuming. The transferred genes have to show a stable expression until tree harvest which may be years to decades after tree planting. Studies on transgene stability have been carried out with *rolC* transformed poplar (Fladung 1999, Kumar & Fladung 2001). The clear phenotype of these transgenics with small, light green leaves allows to detect gene silencing or transgene loss by occurrence of phenotypic reversions in newly developed side shoots or single leaves. General mechanisms causing transgene loss in poplar have not yet been identified, but there are indications that the

presence of inverted T-DNA copies near an inserted transgene may cause physical loss of the transgene during replication. The stability of transgene expression is at least in some cases negatively influenced by integration of the transgene into AT-rich regions which occur in regions of heterochromatin. These are genomic regions, often located near the centromer of a chromosome, which are not expressed. However, field experiments with trees transformed with marker genes showed that, when the transgene is inserted as single copy in euchromatin regions, silencing was a rare event in the investigated poplars (Hawkins et al. 2003, Fladung et al. 2004).

Pollen of trees may be dispersed over long distances and makes introgression of the gene into wildtype populations likely. This process can be monitored by the genetic markers specific for the introgressed transgenes (for further information see Chapter 8 of this book). Since it will not be possible to exclude that the release of a transgene may negatively affect an ecosystem, it is an urgent task to develop sterile trees. In hybrid breeding of crops, male sterile lines are an established tool to avoid self-pollination of the parent plants. Techniques in genetic engineering involve the expression of cytotoxic genes in flower organs by using promoters that are specifically expressed in the tapetum layer of anthers. A different approach is to directly manipulate the transition process from the vegetative to the generative state. A prerequisite for this is knowledge on the genes that control flower formation in trees. The *LEAFY* (*LFY*) gene from *Arabidopsis* encodes a meristem identity factor, which, if put under the control of the 35S promoter, causes early flowering in the monoecious *Arabidopsis* (Mandel & Yanofsky 1995). However, studies with poplar showed that the situation in poplar trees is more complicated, partly due to their dioecious nature. Poplars over-expressing the *Arabidopsis* *LFY* gene showed premature flower formation after 6 months, while wildtype poplars flower after 5 years, but this effect of premature flowering was limited to male plants (Rottmann et al. 2000). Interestingly, overexpression of *PTLF*, the poplar homolog of *LFY*, did only affect the flowering time in 2 out of 19 transgenic poplar lines, but consistently changed flowering time in all transformed *Arabidopsis* lines. Obviously, the poplar gene is under a tighter control, which specifically acts in poplar. Transgenic poplars expressing *PTLF* in antisense will show whether the down-regulation will delay flowering or cause sterility, but the results of these experiments will not be available before 5 years after the start of the experiment.

Conclusion

The production of herbicide-resistant trees exemplifies that genetic engineering projects may be two-edged. The herbicide glyphosate is considered less detrimental to the environment than other herbicides since it is non toxic to humans and biodegradable (Williams et al. 2000, Peterson & Hulting 2004). On the other hand,

herbicide resistant plants and their complementary herbicide are offered by the same company which causes dependency of the forester or farmer on a few companies and reduces the diversity of cultivated plant varieties.

Explicitly positive are approaches to genetically modify trees that use the potential of transgenics to lighten the pollution burden of our environment, e.g. by using trees with low lignin content less chemicals for pulping are needed. Trees with increased biomass production will increase growers' profit (see also Chapters 2 to 4 of this book). This makes sustainable wood production destined as biomass for biofuel or woodchips to be burned in power plants more attractive for farmers (further information in Chapters 5 and 6 of this book).

However, in order to use the clear advantages of transgenic trees in a responsible manner an improvement of our knowledge on the behaviour of the transgenics in the field is necessary and, in addition, more advanced genetically modified trees have to be generated which need to be sterile and have to express the transgene only in the target tissue by using more specific promoters instead of the constitutive 35S promoter.

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Part III – New Biological Methods in Assessment of Wood and Wood Products

8. Molecular Genetic Tools for the Identification of the Origin of Wood

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Introduction

Primary determinants of the value of wood are its dimension as well as its physical and chemical properties. However, additional features apart from those directly related to wood processing and conversion became increasingly important in the recent past. In particular, the management of forests during wood formation became an issue relevant for the trade of wood and wood products on worldwide markets (Bennett 2001). Wood in forests managed against the principles of sustainability can be harvested at lower costs for the forestry enterprise, but marketing of wood or wood products from non-sustainable forestry operations is becoming increasingly difficult. In Europe, the import and sale of tropical timber is greatly promoted by a proof of an origin from a company devoted to the prin-

ciples of sustainability. The certification of wood, wood products, or businesses involved in forestry, wood harvest, and wood processing is of particular importance in this context.

Certification of wood and forestry enterprises

Forest destruction and degradation continue to be main threats to global biodiversity and cause enormous environmental damage in particular in developing countries of the lower latitudes. While the forest area in industrialised nations increased during the last decade, considerable losses of forest cover in developing countries diminish the forested area in all main regions of the tropics (FAO 2001). Efforts to combat deforestation play an important role in global environmental issues and strategies for the development of countries without compromising the maintenance of a healthy environment for the present and future generations (Lanly 1992).

Today, forest certification plays an important role to develop and implement sustainable forest management practices in temperate, boreal and tropical forests. Several agencies independently developed systems to assess the operation of forestry enterprises. The most widely recognised schemes proposed by non-governmental organisations are the certification by the Programme for the Endorsement of Forest Certification Schemes (PEFC; <http://www.pefc.org/internet/html/index.htm>) and, in particular in tropical forests, the Forestry Stewardship Council (FSC; <http://www.fsc.org/en/>) (Cauley et al. 2001).

Different certification schemes have been proposed and were implemented, which share, however, several important characteristics. The objective of forest certification is to identify forestry enterprises working in accordance to commonly accepted principles of sustainable forest management. The assessment of sustainability is not restricted to an observation of the maintenance of the forested area, but is also based on a number of ecological criteria including conservation of biodiversity, appropriate watershed management, erosion control, etc. and may also refer to the socio-economic situation of the directly involved local communities. An enterprise which has been certified as working in accordance to the principles of sustainability has improved options for the marketing of its products, which often can be recognised by the logo of the certifying agency by end-consumers.

A crucial component of any successful and efficient certification scheme is the chain of custody, i.e. the path taken by raw materials, processed materials and products from the forest to the consumer. A proof of the origin of timber or even finished wood products from one or several certified forestry enterprise(s) is necessary to build trust in forest certification by consumers. Attempts to manipulate the system by including wood from non-sustainable forest management in "certified" products became increasingly lucrative with increased marketing potential and commercial success of products from certified forest enterprises

(Cauley et al. 2001; see also website of the Forest Stewardship Council; <http://www.fscoax.org>).

From this point of view, the development of tools to test the origin of wood has considerable scope for practical application within the context of forest certification. A prime objective is the development of methods to check the plausibility of statements concerning the origin of wood from a particular forest enterprise. The observation of genetic traits offers great potential since genetic information at the DNA-level is a non-manipulable and hence trustworthy trait to assess any biological material at various levels.

Molecular genetic tools

Heritable information of all organisms is stored as a sequence of the four bases adenine (A), cytosine (C), guanine (G), and thymine (T) on the DNA. The nuclear DNA of plants is highly variable in size, ranging from less than 10^8 base pairs (bps) to more than 10^{11} bps. In addition, DNA of plants is found in chloroplasts (cpDNA) and mitochondria (mtDNA). Obviously, only a minute fraction of the overall genome can be studied in detail in marker based studies. Variation among DNA sequences is found at various levels both within coding and non-coding regions. Certain regions of the DNA are highly conserved within species, but can be used to assess differentiation patterns among species, genera, or even higher taxonomic levels [see Chapter 9 of this book for details on the specific example of ITS (internal transcribed spacer) regions in clusters of nuclear-encoded ribosomal RNA genes]. Other regions are useful either in isolation or, more frequently, in combination with each other in order to distinguish individuals or groups of related or unrelated individuals. A distinction between individuals is for example possible with highly variable nuclear microsatellite markers (Tautz 1989) or by means of selective amplification of restriction fragments (amplified fragment length polymorphisms = AFLPs, Vos et al. 1995). Maternally inherited chloroplast markers are especially useful in angiosperms to examine large scale geographic distribution patterns within different species (Petit et al. 2002).

The full genetic information of a particular DNA region can be assessed by DNA sequencing. However, sequencing is still rather time-consuming and costly for large-scale application to many organisms. Thus, it is also possible to investigate variation of DNA by an observation of DNA fragments of particular size usually after amplification of DNA by means of PCR (polymerase chain reaction; see Müller 2001 for more details).

Two basic conditions need to be met in order to apply molecular genetic markers to infer the origin of wood: First, protocols need to be developed to extract DNA in sufficient quantity and quality from unprocessed or processed wood. Second, markers need to be identified which are informative with regard to

an identification of the origin of a particular wood sample. Both requirements are discussed in more detail below.

DNA isolation from wood

Freshly-cut wood is a complex plant tissue containing at least in its outer parts both living and dead cells. After felling, the wood dries out and living cells start to die. The DNA in dead plant cells is no longer active, i.e. translated or transcribed, but remains in principle intact.

Isolation of DNA from dead tissue is feasible and possible for extended time periods as has been shown for example for human tissues. "Ancient" DNA has been recovered from human tissues which were thousands of years old (Krings et al. 1999, Hummel 2003), the technology has been used for the identification of historic persons such as Kaspar Hauser and, most famously, the members of the Romanov family (see Hummel 2003), and the investigation of DNA from recently died humans is a key technology in modern forensics. Although much less research has been conducted on plant species, numerous studies proved the possibility to isolate DNA from wood and woody tissue of different age (Dumolin-Lapègue et al. 1999). Isolation of "ancient" DNA from plant material which has been conserved for hundreds or even thousands of years has been reported not only for wood and other macrofossils (Gugerli et al. 2005), but also for pollen (Parducci et al. 2005) and seeds (Jaenicke-Després et al. 2003).

Protocols for the isolation of DNA from wood and other "difficult" plant tissues basically follow procedures established for material which is more suitable for the extraction of DNA, in particular young leaves, but also dormant buds, roots, cambial tissue, and other "living" tissues (Deguilloux et al. 2002). Extraction methods based on the CTAB (cetyl trimethyl ammonium bromide) method (Doyle & Doyle 1987) and other standard protocols for DNA extraction from plant tissues may be applied. However, extraction of DNA from wood is often based on various commercially available kits such as the DNeasy Plant Minikit from Qiagen (Dumolin-Lapègue et al. 1999). Standard protocols are modified in order to increase DNA quantity and quality (Rachmayanti et al. 2006).

Main obstacles for DNA extraction from wood and wood products as compared to other plant tissues are as follows:

- **Mechanical:** Wood is a hard plant tissue and a special mechanical treatment is needed in order to disrupt plant cells such as fibres, vessels and parenchymatic tissue containing intact DNA and DNA fragments. Mechanical pre-treatment of wood by means of drilling or slicing with knives or scalpels is necessary to disrupt cells with thick cell walls. Over-heating of tissue e.g. during drilling must be avoided since it may lead to irreversible degradation of DNA.

- **Chemical:** Numerous agents and wood compounds potentially inhibit DNA extraction or result in low-quality DNA not suitable for amplification by PCR. For example, many phenolic compounds of the lignin metabolism are present in different concentrations in all types of wood; many of them are strong inhibitors for the extraction of high-quality DNA. The success of DNA isolation and subsequent PCR amplification may also be dependent on the tree species. For example, the amplification of cpDNA from *Dryobalanops aromatica* (contains the monoterpene borneol as secondary compound) was less effective than in other members of tree family Dipterocarpaceae (our unpublished results). Many chemicals used for wood treatment are also potential PCR inhibitors. Thus, wood treatment and modification during processing is expected to influence DNA extraction. However, only preliminary studies have been conducted on DNA extraction from treated wood (for example from garden furniture, window frames etc., our unpublished results). The success of DNA isolation of treated and processed wood depends on the type and the intensity of the treatment and has not yet been studied in detail.
- **Biological:** Decomposition of wood by fungi and micro-organisms results in degradation of DNA from the tree and the presence of wood decaying organisms provides an alternative source of DNA. Contamination with DNA from other organisms is expected to be particularly severe on the surface of wood after long periods of storage.
- **Age:** Degeneration of DNA will start after the death of a plant cell. It results in splitting of intact DNA into fragments of small sizes in the range of several hundred base pairs.

In combination, these obstacles result in much lower DNA yields after extraction from wood as compared to isolation from living, soft plant tissue. Furthermore, the extracted DNA is expected to be more degraded, i.e. the average fragment length is expected to be shorter, in particular if the wood has been stored for extended periods of months to years after harvest. Particular efforts are needed to avoid contamination with DNA from other sources (Hummel 2003).

Molecular markers for the identification of the origin of wood

The choice of markers useful for the identification of the origin of wood depends on numerous species-specific differentiation patterns. Thus, only some general guidelines are presented in this section. More details are presented below in the case study on dipterocarps.

The wood of some closely related, mainly tropical species is traded under a single name and the identification of the species with molecular tools is a necessa-

ry prerequisite for further studies on the origin of wood. Species identification confines the potential origin of wood. A clear-cut assignment to a specific geographic region is particularly possible in endemic species (see below).

For single species, informative markers to identify the origin of wood exhibit strong variation among populations, but only limited or ideally no variation within populations. However, typical life history characteristics of trees such as their long life and generation time, overlapping of generations, and efficient means of gene flow in particular by means of pollen result for the majority of species in high variation within populations but only low differentiation among populations (Austerlitz et al. 2000). Consequently, at biparentally inherited gene loci such as microsatellites (= simple sequence repeats; Tautz 1989) or isozymes (Hamrick et al. 1992), the proportion of the total variation due to genetic differentiation among different populations ($G_{ST} = F_{ST}$; = genetic variation among populations divided by the total genetic variation rarely exceeds 10% for the majority of forest plants). Thus, the majority of easily scorable biparentally inherited molecular marker loci are of limited value to identify the geographic origin of wood. Much stronger differentiation as compared to biparentally inherited markers has been observed for various angiosperm species at maternally inherited traits. Mitochondrial DNA (mtDNA) is maternally inherited in almost all plants and animals (Avice 2000). Chloroplast DNA (cpDNA) is also maternally inherited in most angiosperms (Dumolin et al. 1995), but shows paternal inheritance in gymnosperms (Neale & Sederoff 1989). Since pollen movement is more wide-spread as compared to the movement of seeds in most species, markers which are not effected by pollen movement, i.e. maternally inherited markers, often show higher differentiation (Petit et al. 2003).

The spatial distribution of cpDNA haplotypes has been studied in much detail for European oaks (*Quercus* spp.). Most of the variation is found among different populations ($F_{ST}=0.85$) with considerable differences among cpDNA haplotypes from different glacial refugia (Petit et al. 2002). Thus, a clear geographical pattern among naturally regenerated oak populations has been observed in Europe. The identification of the origin of wood by means of molecular genetic markers is well-advanced for European oaks of the section *Lepidobalanus*, in particular *Quercus petraea* and *Quercus robur* (Deguilloux et al. 2003).

Patterns of natural genetic differentiation among different populations of forest trees have been shaped by the joint effects of evolutionary factors. Thus, they are an outcome of population history, which has not only been influenced by natural factors, but also by human impact. In particular, movement of forest reproductive material of plantation species has considerably modified genetic structures at marker loci. For example, almost complete differentiation between cpDNA haplotypes of *Dalbergia sissoo* was observed between natural populations and plantations of the species in Nepal (Pandey et al. 2004). Thus, the plantations did not originate from any of the investigated natural populations. Most likely,

reproductive material was transported over long distances during plantation establishment.

Most forest trees have not been “domesticated”, and variation patterns at marker loci reflect both natural processes and usually unintentional human impact to variable degrees. Breeding programmes have been conducted for a comparably small number of plantation species only. The identification of material from plantations established with material from intensive breeding programmes is expected to be more straightforward, especially if only a limited number of clones have been planted. The labelling of genetically modified trees grown in intensively managed plantations by an easily observable markers is a long-term perspective for the application of molecular tools to assess the origin of wood (see Chapters 4 and 7 of this book for further reading).

For the majority of the species, only few markers show variation patterns which make them useful to identify the origin of the wood. Differentiation patterns allow to identify species and individuals or clones, but rarely provenances, single stands or an area under the management of a particular company. Nevertheless, “informative” markers can be identified at various levels from large provenance regions to smaller subunits of populations for most species, if large scale inventories involving many samples and populations are undertaken. Both putatively selective markers coding for functional genes and neutral markers without a known function are in principle suitable for purposes of the identification of the origin of wood, as long as they are “informative” with regard to the observation of relevant spatial variation patterns. In many cases, only a combination of several informative markers will allow to come to a reliable conclusion with regard to the putative origin of tested wood (Petit et al. 2002).

Maternally inherited markers (mtDNA or, in case of angiosperms, cpDNA markers) are particularly useful due to their frequently higher levels of population differentiation. The isolation of “cpDNA” from plant tissue not containing active chloroplasts such as wood has been shown to be feasible since “cpDNA” is present in proplastids and all types of plastids developing from them, and thus not restricted to chloroplasts of photosynthetically active, green plant cells.

DNA extraction from wood often results in rather short fragments of a few hundred base pairs in length due to the high degree of degeneration of DNA in most wood samples (see above). Longer fragments cannot be amplified by PCR if DNA has been extracted from wood. Thus, short DNA fragments showing strong differentiation among populations such as many chloroplast microsatellites (Vendramin et al. 1996, Weising & Gardner 1999, Sebastiani et al. 2004) are among the potentially most informative markers for the identification of the origin of wood.

A case study: Tropical dipterocarps

Importance of dipterocarps in Southeast-Asian forests

Most tropical forests in Southeast-Asia are dominated by a single species-rich tree family, the dipterocarps (Dipterocarpaceae). The family is pantropical with comparatively few species occurring in the neotropics and in Africa. The subfamily Dipterocarpoideae is very species-rich and common in Asian evergreen and Monsoon forests (see Chapter 3 of this book). The centre of species diversity is reached in Borneo (Kalimantan) with more than 260 described species; few species are native to forests east of the Wallace-Line that constitutes a major break in flora and fauna between Asia and Australia in the Malay archipelago.

In many Asian forests, which are regarded as a centre of global biodiversity, more than 50% of all trees including the majority of emergents and trees of the canopy are dipterocarps. Dipterocarps are not only a key resource in particular in tropical Southeast-Asia (Whitmore 1975), but they are also among the most important tropical timbers for trading (trade names: meranti, balau for *Shorea* spp., keruing for *Dipterocarpus* spp., kapur for *Dryobalanops*, etc.). In many regions, dipterocarps are critically endangered due to forest destruction and non-sustainable forest management leaving only secondary forests of little commercial value after logging (Appanah & Turnbull 1998).

Sustainable management of dipterocarp forests is feasible, if harvesting is carefully controlled and natural regeneration promoted (Lamprecht 1986). Thus, the development of tools to identify dipterocarp wood from sustainably managed forests will contribute to the application of sustainable management practices and the conservation of dipterocarps and their associated species. The large number of commercial species needs to be taken into consideration for the development of tools for wood identification. The wood from more than 100 species belonging to the species-rich genus *Shorea* is differentiated into only a few trade names (white meranti, yellow meranti, dark red and light red meranti; see Appanah & Turnbull 1998).

DNA isolation from dipterocarp wood

There are some major issues in order to develop molecular markers for the certification (identification of falsification) of the origin of wood from dipterocarps, i.e. (1) the adaptation of DNA extraction methods for marker analysis from wood, (2) the development and application of different markers – chloroplast and nuclear markers – that showed a strong differentiation between geographic origins, (3) the development of specific PCR primers in order to amplify very short polymorphic regions in degraded DNA probes from wood samples.

In our laboratory, DNA isolation methods were optimised in wood samples from natural populations and from wood enterprises (Rachmayanti et al. 2006). A

summary of the optimised DNA isolation method from dipterocarp wood is described in Table 1. Any surfaces of the wood sample that have potentially been contaminated by cellular material of another individual has to be removed. In order to prepare the wood sample for DNA extraction, it should be cut into minute pieces using a sterile scalpel and pulverised using a grinding mill such as the Mixer Mill from Retsch. In order to avoid sample overheat it is necessary to freeze the sample in liquid nitrogen before grinding. Commercially available DNA extraction kits such as the DNeasy Plant Mini Kit and the MagAttract 96 DNA plant core kit from Qiagen are regularly applied in our laboratory. Dependent on the wood sample (wood age, species, heartwood, bark etc.), the extraction protocol such as type of lysis buffer, duration of incubation etc. was modified. Adding of PVP (polyvinylpyrrolidone) into the lysis buffer was the most frequent modification needed.

DNA of sufficient quality for PCR amplification was isolated from wood of *Shorea* species with this method. Fig. 1A shows the PCR results of three different cpDNA regions of different length. High quality DNA isolated from leaves of the

Table 1 Summary of the optimised DNA isolation method from dipterocarp wood

Wood Preparation	Clean inner parts of wood are taken after removal of the surface area and cut into small pieces using a sterile scalpel
Wood quantity	50-100 mg
Wood powdering	
1. Beads	Add one stainless steel bead (Ø 5 mm) per 2 ml microcentrifuge tube
2. Freezing	Incubate wood material for 5 min in liquid nitrogen prior to each grinding
3. Grinding	Grind 2x 5 min at defined speed (e.g. at 70 units of Mixer Mill Type MM2 from Retsch)
Lysis procedure	
1. Buffer	Add PVP to the lysis buffer AP1 (DNeasy Plant Mini Kit, Qiagen) up to 3.3% (w/v)
2. Incubations	Incubate over night at 65 °C with shaking or rotating Incubate at - 20 °C for 15 min after adding of AP2 buffer (DNeasy Plant Mini Kit, Qiagen)
Extraction of DNA* (alternative procedures)	1. Spin-column procedure of DNeasy Plant Mini Kit from Qiagen 2. Magnetic-based procedure of MagAttract 96 Plant Core Kit from Qiagen, thereby using a magnetic particle concentrator from Dynal Biotech
Elution of DNA*	The second eluate (DNeasy Plant Mini Kit method, Qiagen) is collected separately from the first eluate and applied in the PCR

* For further details see instructions by the manufactures of the kits.

same individuals was used as a positive control in the PCR. A reaction mixture without DNA (DNA was replaced with HPLC water) was used as a negative control in the PCR. The success of the amplification was dependent on the amplified cpDNA fragment (length of the amplicon) and the dilution of the isolated DNA prior to PCR. PVP was very effective to reduce inhibitory substances. A PCR inhibitor test where DNA eluate from wood was added to high quality DNA confirmed the improved DNA quality (Fig. 1B).

Differentiation of dipterocarps at marker genes: Interspecific variation - phylogenetic analysis

Phylogenetic studies of dipterocarps have been performed using both nuclear gene markers and chloroplast markers. Most authors used the total sequence information of specific chloroplast genes or intergenic regions to unravel the phylogenetic relationships of asian dipterocarps. DNA sequences were studied at the *rbcL* gene (Dayanandan et al. 1999, Morton et al. 1999), non-coding regions of the *trnL* intron, the *trnL-trnF* intergenic spacer region (Gamage et al. 2003, Kamiya et

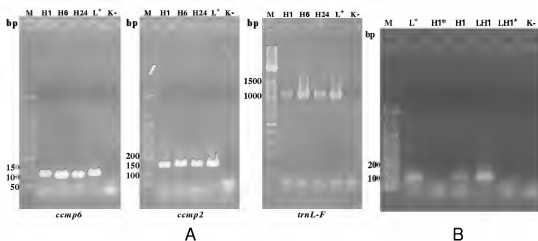


Fig. 1 A. PCR profiles of *Shorea* DNA samples amplified with primers *trnL-F* (Taberlet et al., 1991), *ccmp2* and *ccmp6* (Weising & Gardner 1999). The lengths of the cpDNA fragment amplified by primers *ccmp6*, *ccmp2* and *trnL-F* were about 0.1, 0.15 and 1.1 kb, respectively. M = DNA size standard; H1, H6 and H24 = wood DNA of meranti (botanical name unknown), *Shorea leprosula* and *Shorea ovalis*, respectively; L⁺ = positive control (leaf DNA); K⁻ = negative control (water). B. Inhibitory test: Effect of PVP addition during DNA isolation on subsequent PCR amplification with *ccmp6*. M = DNA size standard; L⁺: leaf DNA; H1* = wood DNA of meranti isolated without PVP addition; H1 = DNA from the same wood (H1*) isolated with PVP addition; LH1 = leaf DNA (L⁺) mixed with an equal volume of H1 DNA; LH1* = leaf DNA (L⁺) mixed with an equal volume of H1* DNA; K⁻ = negative control of water

al. 1998) and at the *matK* gene (Kajita et al. 1998, Li et al. 2004). Some studies have applied PCR-RFLPs (PCR-restriction fragment length polymorphisms) of chloroplast regions covering several genes and intergenic regions. PCR-RFLP involves the PCR amplification of specific genomic regions with conserved primers and subsequent restriction of the fragments with restriction enzymes. The PCR-RFLP method has been applied on 11 specific genes by Tsumura et al. (1996) and on five genes/intergenic spacer regions plus three chloroplast microsatellites by Indrioko et al. (2006). In the latter study 129 trees belonging to 58 species in 9 genera, i.e. *Anisoptera*, *Cotylelobium*, *Dipterocarpus*, *Dryobalanops*, *Hopea*, *Shorea*, *Parashorea*, *Vatica* and *Upuna*, have been analysed. No variation within species (up to 4 samples per species have been analysed) has been detected for any of the cpDNA markers analysed. The combination of different markers and/or sequence information allows an unambiguous identification of the species. Species identification is one prerequisite to test the correctness of declaration of wood from unknown origin. Especially for species with relatively restricted geographic range (i.e. for endemic species) this information is highly valuable and allows the detection of wrongly declared wood origins, or in specific cases (for example for endemic species or in case of a locally restricted genotype) a verification of the origin. For example, the endemic species *Upuna borneensis*, *Shorea fallax* and *Anisoptera reticulata* of northern Borneo were characterised by several diagnostic (species-specific) cpDNA markers (Indrioko et al. 2006).

A cpDNA phylogenetic tree (Indrioko et al. 2006) does not reflect the grouping according to wood characteristics (balau, selangan batu, white meranti, red meranti and yellow meranti) within the important genus *Shorea*. However, sequencing of the nuclear *PgiC* (phosphoglucose isomerase) gene in *Shorea* revealed that species belonging to each of the three timber groups balau, yellow meranti, and red meranti formed one specific (monophyletic) clade within *Shorea* (Kamiya et al. 2005). Additional information on variation in nuclear gene markers is available for genus *Shorea* for the *GapC* (glyceraldehyde-3-phosphate dehydrogenase) region (Ishiyama et al. 2004) and the ITS regions (K.S. Yulita & R.J. Bayer, unpublished; see Chapter 9 for a general explanation of the ITS regions). This sequence information will be useful in the future to detect sequence variation that distinguishes between species and different geographic origins within species (see below).

Differentiation of dipterocarps at marker genes: Variation between geographic regions

Chloroplast markers often show low variation within populations or larger geographic regions, but strong and significant differentiation between regions. However, preliminary studies of cpDNA variation in *Shorea* species showed no or low variation within species. Larger surveys of cpDNA variation were performed in *Shorea leprosula* and *Shorea parvifolia* as the most common and widespread trees of

lowland dipterocarp forest in Indonesia. Virtually no variation was detected in seven geographically distinct regions of *S. leprosula*, but two haplotypes were distinguished in seven *S. parvifolia* populations, one of which is specific for region Sari Bumi Kusuma in Borneo. The two haplotypes differ in length by only one base pair in the chloroplast microsatellite *comp6*.

In order to obtain additional information, the AFLP marker technique (Vos et al. 1995) as a very effective, fast and reliable tool to reveal restriction fragment polymorphisms was applied in the same *S. parvifolia* and *S. leprosula* populations from Borneo and from Sumatra. *S. leprosula* ($G_{ST} = 0.25$) and especially *S. parvifolia* ($G_{ST} = 0.31$) revealed quite high differentiation between populations at AFLP markers. This became even more apparent, when G_{ST} values for individual AFLP markers are calculated showing a maximum G_{ST} of 0.77 for *S. parvifolia* and a maximum G_{ST} of 0.72 for *S. parvifolia*. In AMOVAs (analyses of molecular variance) at 3 hierarchical levels based on the geographical regions (islands) performed separately for *S. leprosula* and *S. parvifolia*, a significant proportion (26.2%) of the total variation in *S. leprosula* was attributable to differences between islands, while variation between islands was not significant (10.9%, $P = 0.073$) in *S. parvifolia* (Cao et al. 2006).

The same pattern is also reflected in frequency distributions of two highly differentiating AFLP bands (Fig. 2). The AFLP marker that is shown in the upper panel of Fig. 2 has a frequency of 96% in population Sari Bumi Kusuma (SB) in Borneo and is absent in nearly all other regions. Note that population Sari Bumi Kusuma is also characterised by a different chloroplast type (see above) in *S. parvifolia*. The variation pattern corresponds with the pattern found at cpDNA marker *comp6*. The AFLP marker in the lower panel of Fig. 2 is present in all samples from Borneo but virtually absent in Sumatra. It should be mentioned that this is a quite surprising pattern for nuclear AFLP markers that usually have a low G_{ST} value (LaraGomez et al. 2005).

In future studies, we will characterise those AFLP fragments by sequencing that show strong differentiation between regions. Sequence information for different regions of the genome will help to generate PCR markers that allow for the amplification of short informative regions from degraded wood DNA.

Conclusions and perspectives

The development of molecular genetic tools to identify the origin of wood has great potential for application in the context of forest certification and the control of the global trade with timber. The need to develop non-manipulable tools to identify the origin of wood and wood products is greatest for tropical timber. All key technologies such as DNA extraction from wood and investigation of informative DNA regions are now developed to use in "genetic fingerprint" techniques for wood identification. However, problems are encountered in particular for

tropical species in view of the numerous tropical timber species traded on a regional or global scale and the lack of knowledge on patterns of genetic variation at marker loci for most species.

The methods outlined in this chapter are not the only tools to infer the origin of wood. “Genetic fingerprinting” techniques need to be combined with other methods including studies on wood anatomy and chemistry as well as the use of microscopic techniques such as FTIR (Fourier transform infrared) imaging (see Chapter 10 of this book) and stable isotopes (Forstel & Boner 2003) in order to

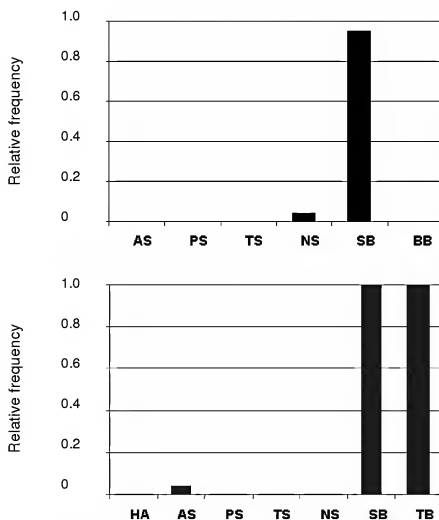


Fig. 2 Variation patterns of two AFLP markers showing the relative marker (band) frequencies in different populations. Upper panel: *Shorea parvitolia*. AFLP marker that is specific for population Sari Bumi Kusuma (SB) in Borneo. Lower panel: *Shorea leprosula*. AFLP marker with high frequency in Borneo (populations BB, SB and TB) that is virtually absent in populations from Sumatra (HA, AS, PS, TS, NS)

develop cost-efficient and highly reliable tools to infer the origin of wood. However, molecular genetic markers are expected to play an important role for the identification of the origin of wood, in particular because genetic traits are stable since environmental conditions have no impact on variation patterns.

A positive proof of the exact origin of wood will not be feasible for many species in the near future since no detailed information is available on spatial variation patterns on "informative" gene markers that cover the whole distribution range of the species. Even a combination of several markers will only allow to identify comparatively large geographic regions from where wood originated from. However, molecular markers will certainly become a reliable tool to test statements concerning the origin of wood. False declarations of the origin of wood can be recognised as such. This will greatly facilitate the efficient control of global trade with wood and wood products, promote the marketing of timber from certified forests, and thus eventually contribute to the sustainable management and the long-term conservation of forest resources and biodiversity.

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9. Molecular Detection of Fungi in Wood

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Introduction

Wood is mainly composed of cellulose and lignin. These polymers are the two most abundant organic substances in the terrestrial environment and provide a huge reservoir of carbon- and nitrogen-containing compounds for living organisms (Kalusche 1996, Aro et al. 2005). However, in order to access this reservoir, an organism needs to be capable of breaking down the polymers. The primary decomposers of wood are wood-decaying fungi, mainly white and brown rot fungi (Hibbett & Donoghue 2001).

Early detection of fungi colonising wood can be of great economical importance in living trees as well as in wood in use. Although naturally or artificially fungal infected wood may still be used for special applications (Mai et al. 2004),

infected wood needs to be separated from healthy wood in order to prevent losses in downstream processing.

Taxonomical identification of decay fungi in wood which is already in service is also of great interest. Wood is amongst the most ancient material (Blanchette 2000) and is still of significant importance in historical buildings and cultural assets as well as in present construction, indoors as outdoors. Decay of wood used in construction leads to reduced stability and obviously presents a serious danger (Huckfeldt et al. 2005). For safety and conservation reasons, therefore, it is necessary to identify the presence of incipient decay in such structures for control and/or remediation or replacement purposes. Knowledge about the identity of the fungi causing the decay becomes all the more important when new control strategies, based on the abiotic and biotic requirements of the fungi (e.g. environmental control), are to be applied (Palfreyman et al. 1995; see also Chapters 13 and 14 of this book). Finally, detection and identification of fungal species is a prerequisite in studies addressing fungal ecology and biology (e.g. Heilmann-Clausen & Christensen 2005, Jonsson et al. 2005).

Methods for detection of fungi in wood

Several fundamentally different methods have been developed to assess the presence of fungi in wood. Each method has its advantages and disadvantages concerning their ease of use, sensitivity, specificity, reliability, technical requirements, speed, costs and operating expenses.

Visual inspection of wood for presence of fruiting bodies, mycelium with or without vegetative spores or decay patterns is only useful for detecting advanced decay or abundant presence of fungal biomass (Schwarze et al. 2000). In most instances, identification of the fungal species in the absence of fruiting bodies or spores is almost impossible. Fruiting bodies and spores are only differentiated in response to specific environmental conditions and some species may not be able to produce fruiting bodies and spores at all (Breitenbach & Kränzlin 1984-2000, Jahn 1990, Ellis & Ellis 1998, Huckfeldt 2000).

Isolation and culturing of fungi from inhabited wood is more sensitive than the pure visual inspection. Fungal identification based on growth characteristics on different culture media and, possibly, mycelial spore formation (Stalpers 1978, von Arx 1981, Kim et al. 2005), however, is rather difficult even for a trained mycologist (Fig. 1). Another inherent drawback of this method is that not all species living in wood might be cultivable (at least not on the limited number of media normally used) or are overgrown by others during isolation and thereby escape detection (Johannesson & Srenlid 1999).

Methods not depending on the presence of fruiting bodies, spore formation, or cultivation have been developed to enable a less biased analysis. The identification with fatty acid methyl esters (FAME) and fatty acid and sterol (FAST)

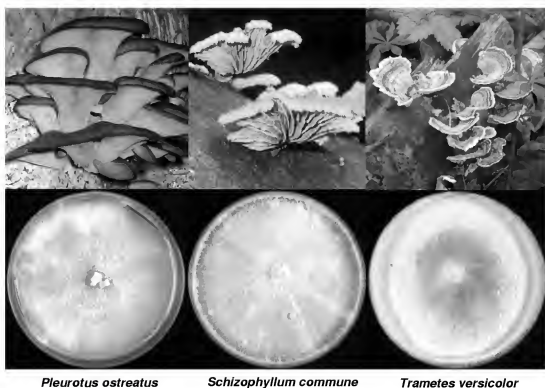


Fig. 1 Fruiting bodies (upper row) and pure cultures (lower row) of three white-rot basidiomycetes. Whilst the fruiting bodies are easily distinguishable, the isolated fungal cultures demonstrate how similar mycelium of different species can be. Photos taken by M. Rühl and P. Hoegger

profiles by gas chromatography is based on the presence of specific fatty acids ("signature fatty acids") and sterols and their relative amounts in a particular species (Bentivenga & Morton 1994, Müller et al. 1994). The profiles have to be compared to a database, commercially available from MIDI Inc. (Newark, DE, USA). However, this database covers only a limited selection of fungal species (mainly yeasts and moulds) and does not contain any profiles from wood decay fungi. Diehl et al. (2003) attempted to build a database for wood decay fungi, however, they encountered problems with fungal cultivation and fatty acid extraction leading to weak matches in species identification.

More recently, a method analysing fungal mycelia by MALDI-TOF MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) without extraction of any specific group of organic compounds was presented (Schmidt & Kallow 2005). Mass spectra of whole cells are obtained from a collectivity of compounds such as proteins, cell wall and membrane components. Comparing the peak patterns of the mass spectra, mycelia of the closely related species of wood decay fungi analysed in the study (*Serpula lacrymans*, *Serpula*

himantoides, *Coniophora puteana*, *Coniophora marmorata*, *Antrodia vaillantii* and *Antrodia sinuosa*) could be differentiated. Fourier transform infrared (FTIR) spectroscopy is another method for identifying fungi by recording IR spectra of whole cell compounds (Liu et al. 2004, Naumann et al. 2005, Chapter 10 of this book). FTIR spectroscopy combined with microscopy and imaging was reported to be applicable in biological materials (Salzer et al. 2000) and its potential for the specific detection of fungi in wood was shown recently (Naumann et al. 2005, Chapter 10 of this book). Once a database for FTIR spectra of fungi in wood has been established, this method will prove helpful for the detailed analysis of the biology and mechanisms of fungal hyphae growing in wood.

Analysis of volatile organic compounds (VOCs) by electronic sensor devices is also considered useful to detect fungi growing in and on wood (Wilkins et al. 2003, Kuske et al. 2005, Chapter 11 of this book). As a special advantage, VOC detection enables to inspect valuable objects that can or should not be sampled destructively, which in contrast is necessary for almost all other methods. The identification of characteristic VOCs, however, is a prerequisite for establishing such a tool. For wood decay fungi and moulds, this is only at an initial stage (Ewen et al. 2004, Schleibinger et al. 2005).

The usefulness of all these methods depends on the stability and heritability of profiles in the organisms being analysed and has to be determined empirically for each group of study organisms (Bentivenga & Morton 1996). FAME and MALDI-TOF mass spectra were shown to vary greatly with physiological changes in the organism (Bentivenga & Morton 1994, Schmidt & Kallow 2005), FTIR spectra with different fungal cell types (Liu et al. 2005), and VOCs with strains and growth substrates (Schleibinger et al. 2005). Therefore, these methods have to be carefully evaluated for direct identification of fungi growing in wood due to the various factors influencing the fungal physiology (environmental conditions, wood species, treatment of wood, etc.).

Various immunological assays have been developed to detect fungal colonisation of wood using monoclonal and polyclonal antibodies. Selection of the antibody type depends on the required specificity for the detection. Polyclonal antibodies recognise multiple chemical sites (epitopes) and may therefore detect a whole group of species (Toft 1993). If species specific detection is required, monoclonal antibodies, recognising only a single epitope should be used (Burge et al. 1994). Antibodies can be used in different detection assays depending on the respective requirements. Particle agglutination tests are simple, rapid, and fairly portable, however, not quantitative nor can they be automated (Clausen 2003). Dot blot assays are sensitive and quantitative, but not simple, rapid, nor portable and can also not be automated (Clausen et al. 1991). Enzyme-linked immunosorbent assay (ELISA) provides sensitive, quantitative and automated measurements allowing high sample throughput (Clausen et al. 1991, Kim et al. 1991). Detection by chromatographic immunoassays combines the speed of antibody-mediated par-

ticle agglutination and sensitivity and specificity of ELISA, though it is not suited for large sample numbers. Clausen and Green (1996) developed and patented a test for decay fungi in wood that can easily be used in the field. In order to detect specific fungi or their enzymes or metabolites *in situ*, immunolabelling methods can be used in conjunction with light, electron, or confocal laser scanning microscopy (Clausen 1997, Daniel et al. 1991, Xiao et al. 1999). Immunological methods have the potential to differentiate between live (active) and dormant or dead mycelia depending on the antigens that are recognised. The specific recognition of (secreted) enzymes may be useful to detect only live, actively growing fungal biomasses, as for instance in the case of the plant pathogen *Gaeumannomyces graminis* var. *tritici* growing in plant tissue (Thornton et al. 1997).

Among the techniques for DNA analysis, polymerase chain reaction (PCR)-based methods are best suited for the detection of fungi in wood. Through PCR-amplification even minute amounts of DNA can be analysed, thereby providing high sensitivity. Random amplified polymorphic DNA (RAPD) analysis was reported for the identification of *S. lacrymans* (Schmidt & Moreth 1998). However, a major drawback is the need of pure fungal cultures for analysis and RAPD analysis is prone to contamination by any kind of DNA. Specific PCR primers have been developed to identify the sapstain fungi *Ophiostoma piceae* and *Ophiostoma quercus* (Kim et al. 1999), the brown rots *Coniophora eremophila* and *A. sinuosa* (Demetriou et al. 2000), and the root and butt rots *Heterobasidium annosum sensu stricto* and *Heterobasidion parviporum* (Hantula & Vainio 2003) in wood. Although this approach prevents contamination, it needs to be established for each species or group of species to be investigated. Due to the availability of universal PCR primers (White et al. 1999), analysis of the nuclear ribosomal DNA (rDNA) is the most often used approach for identification of fungi. Amplified rDNA fragments (amplicons) can be separated by denaturing gradient gel electrophoresis (DGGE) and compared with fragments from reference strains (Vainio and Hantula 2000). This method circumvents the need of a cloning step for samples with more than one species, but it offers only a limited resolution. The amplicons can also be typed by digestion with restriction enzymes to yield specific patterns based on restriction fragment length polymorphisms (RFLPs) which can be compared with reference strains (Johannesson & Stenlid 1999, Schmidt & Moreth 1999, Jasalavich et al. 2000). However, the most complete information on fragment identity is obtained by simply sequencing it (Schmidt & Moreth 2002, Kim et al. 1999, Kim et al. 2005). Obtained sequences can then be compared with the readily available sequence databases (see below). This method does not need any extra reference strains provided the sequence in question is present in a suitable database and is known to be correctly assigned. Identification of fungi by defined sequences is then most reliable. Moreover, the use of real-time PCR, a method detecting the accumulation of amplicon during the reaction, also allows quantitative detection of fungi in wood (Hietala et al. 2003, Eikenes et al. 2005).

Organisation and evolution of rDNA

Ribosomes are structures within cells that direct protein synthesis by translating messenger RNA. They are composed of a small and a large subunit, each comprising different proteins and ribosomal RNAs (rRNAs). The small subunit (SSU) of non-organellar, eukaryotic ribosomes contains the 18S rRNA (designated by its "S-value" which indicates the rate of sedimentation in an ultracentrifuge) and approximately 33 proteins. The large subunit (LSU) contains 3 types of rRNA (5S, 5.8S, and 25-28S) and approximately 49 proteins (Wool et al. 1995, Venema & Tollervey 1999).

The rRNAs of eukaryotes are encoded by highly conserved genes which are typically present in the nuclear genome in several hundred, tandemly repeated copies (Long & Dawid 1980). Each gene is separated from the next by a non-transcribed region known as the intergenic spacer (IGS) region (Fig. 2). This region harbours the greatest amount of sequence variation and different patterns of organisation can be found in different groups of fungi. In nearly all basidiomycetes and several ascomycetes, the IGS is divided in IGS 1 and IGS 2 by the 5S rRNA gene (Fig. 2, Drouin & Moniz de Sá 1995). In most filamentous ascomycetes and most other eukaryotes the IGS is not interrupted and the 5S rRNA gene is found elsewhere in the nuclear genome (Wolters & Erdmann 1989, Drouin & Moniz de Sá 1995).

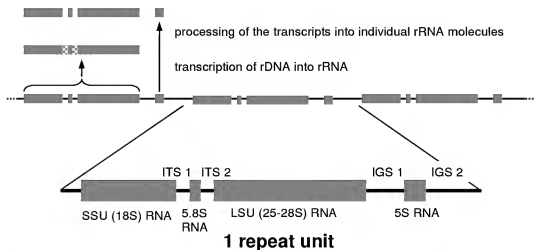


Fig. 2 Organisation of the nuclear-encoded ribosomal RNA genes (rDNA) of fungi. The genes exist as a multiple-copy gene family comprised of highly similar DNA sequences, present in up to several hundred copies. Each repeat unit (typically from 8-12 kb each) has coding regions for one major transcript (containing the primary rRNAs for a single ribosome), interrupted by one or two intergenic spacer (IGS) regions depending on the presence of the 5S rRNA gene

The SSU, LSU and 5.8S rRNAs are transcribed as a single primary transcript which is then cleaved during processing in the nucleus into the three final molecules (Fig. 2). The three genes are separated by two internal transcribed spacers (ITS 1 and ITS 2) which contain signals for processing the primary transcript (Hillis and Dixon 1991). Transcription of the 5S rRNA gene is separate from the three other genes.

The combination of non-transcribed, transcribed "non-coding", and transcribed "coding" regions in the rDNA make it very useful for phylogenetic studies because they evolve at different rates due to distinct selection pressure. The nuclear SSU gene is among the slowest evolving sequences found throughout living organisms and is therefore very useful to study ancient evolutionary events. Similarly, the nuclear LSU gene can be used for ancient comparisons. However, next to highly conserved regions it also contains divergent regions, also known as divergent domains or expansion segments (Houssana et al. 1984). These divergent regions are useful to infer phylogeny on the level of genus/family (Hopple & Vilgalys 1999). The 5.8S and 5S rRNA genes of eukaryotes are similar to the SSU rRNA gene in their useful phylogenetic range (i.e. ancient evolutionary events), however, the small size of these genes restricts their usefulness considerably (Halanynch 1991). Compared to the "coding" rDNA regions, the ITS sequences are more variable. They were shown to be very useful for molecular identification of fungi, as they are often highly variable among distinct species but show only low intraspecific variation (Gardes et al. 1991, Lee & Taylor 1992). Hillis & Dixon (1991) aligned the sequences of all rRNA genes (1 sequence per species) from a selection of taxa of varying relationship. Based on their analysis, one can identify the rDNA regions that are best suited for a given phylogenetic study. The regions within a similarity range from 70 to 90% are most likely to be useful for phylogenetic analysis.

It has become well accepted that rRNA genes of a species were not evolving independently, but in concert, i.e. they undergo a process that homogenises their DNA sequences (Arnheim et al. 1980, Hillis & Dixon 1991, Nei et al. 1997). The multiple copies of rRNA genes within an individual or species, therefore, are usually more similar (or identical) to each other, whereas differences among species accumulate more rapidly. This was shown for example for the rRNA genes of human and apes (Arnheim et al. 1980). The high degree of sequence homogeneity is thought to be achieved by unequal crossing over (interlocus recombination) or gene conversion (Hillis & Dixon 1991, Nei et al. 1997). More recently, it was shown that the 5S rRNA multigene family of filamentous fungi do not undergo concerted evolution but rather evolution by birth-and-death (Rooney & Ward 2005). Nevertheless, even with concerted evolution, some intragenomic variation is possible and sometimes even necessary - for instance, structurally and functionally heterogeneous rRNA gene copies are required at different developmental stages of protists (Rooney 2004). Within basidiomycete fungi, two to five different

ITS types within a single strain have been reported in *Trichaptum abietinum* and in species of the genera *Ganoderma*, *Lecanium*, *Polyporus*, and *Trametes* (Ko & Jung 2002, Kaserud & Schumacher 2003, den Bakker et al. 2004, Wang & Yao 2005, Naumann et al. 2007). In conclusion, whilst gene homogenisation appears to be the prevailing rule, the occasional intragenomic variation should be kept in mind when using rDNA for phylogenetic analysis (Hillis & Dixon 1991).

DNA extraction

In order to carry out molecular genetic analysis of wood decay fungi, their DNA has to be made accessible, i.e. the DNA needs to be extracted and purified from the fungal cells. Various protocols have been developed for isolating DNA from pure cultures of many different fungal species, optimising yield, time, costs, or purity. Most problems encountered during DNA extraction from filamentous fungi are caused by the large amounts of polysaccharides and glycoprotein compounds found in the cell wall of many species. These problems have been overcome by the use of the cationic detergent cetyltrimethyl ammonium bromide (CTAB) and β -mercaptoethanol (Talbot 2001). A robust and quick method that proved very successful in obtaining DNA of sufficient quantity and quality from various filamentous asco- and basidiomycetes (including wood decay fungi) was presented by Zolan & Pukkila (1986).

Often, however, it may be necessary to extract fungal DNA directly from wood samples necessitating the application of more specialised extraction methods. These methods aim to exclude or at least reduce compounds such as polyphenols, tannins, or resin acids present in wood since they are potent inhibitors in downstream reactions on DNA involving enzymes. Bahnweg et al. (1998) presented an optimised protocol for DNA extraction from recalcitrant materials such as wood based on the following main steps: i) tannins are precipitated by CaCl_2 and polyphenols are extracted by methanol prior to DNA solubilisation, ii) DNA extraction is carried out at 4 °C with CTAB and employing the highly toxic benzyl chloride as denaturing agent, iii) Nucleon PhytoPure resin (Amersham Pharmacia, Braunschweig, Germany) is used to remove acid polysaccharides, and iv) DNA is washed with ethanol and chloroform (Bahnweg et al. 1998, 2002). Although this method yields pure DNA without any inhibiting compounds, it also is rather time consuming and expensive. A very quick but also crude method releasing DNA from fungal cells by microwave heating was used for spore samples collected directly from conifer wood infected with sapstain fungi (Kim et al. 1999). Although maybe useful for fungi sporulating on the surface of wood, the method restricts the range of fungal species that can be detected considerably as it relies on spore formation. Another method was developed for molecular analysis of fungi in wood from Norway spruce (Vainio & Hantula 2000). After an extensive number of extractions (5x phenol/chloroform/isoamyl alcohol and 2x chloroform/isoamyl alcohol) this method uses a commercial DNA purification kit, raising the

costs considerably. Based on our experience, a modified protocol provided by Jasalavich et al. (2000) with wood powder and fine sawdust with less numbers of organic extractions and simple DNA precipitation yields satisfactory results whilst not being too time consuming nor expensive (Hoegger et al. 2006). Irrespective of the method used for fungal DNA extraction from wood samples, appropriate controls have to be performed in downstream reactions in order to evaluate the inhibitory capacity of the extract on enzymes.

rDNA analysis as a tool for identification

As explained above, the ITS region of rDNA has been shown to be particularly useful for the molecular identification of fungi due to its proper evolutionary rate. The high sequence conservation of the flanking SSU and LSU rRNA genes make them ideal targets for DNA primer sites to amplify the complete ITS region (including the 5.8S rRNA) by PCR. Due to the high conservation, such primers will amplify the target DNA from many species, thus making them "universal". White et al. (1990) designed such universal PCR primers (ITS1 to ITS4) for the fungal ITS region (Fig. 3, Table 1). Although primers ITS1 and ITS4 became widely used in fungal studies, one has to consider that they are not effectively excluding all plant sequences when dealing with mixed samples of fungi and plants. From our own experience, we know that these primers will not amplify gymnosperm DNA, however, they can amplify angiosperm DNA extracted from wood if there is no or only minor amounts of fungal biomass present. In order to amplify fungal and basidiomycete DNA from mixed DNA samples more specifically, Gardes & Bruns (1993) constructed primers ITS1-F and ITS4-B (Fig. 3, Table 1). More recently, another primer set specifically amplifying all Dikaryomycota sequences

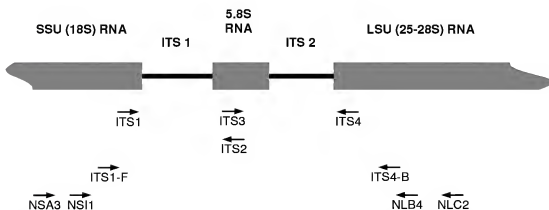


Fig. 3 Positions of primers commonly used for amplification of the fungal ITS1-5.8S RNA-ITS2 region. Sequences and references for primers are given in Table 1

Table 1 PCR primers for the amplification of the ITS1-5.8S RNA-ITS2 region in fungi. For localisation of primers in rDNA see Fig. 2.

Primer	Sequence (5' to 3')	Remark	Reference ¹
ITS1	TCCGTAGGTGAACCTGCGG	Broad range specificity (fungi, plants, protists, animals)	1
ITS2	GCTGCGTTCTTCATCGATGC	Broad range specificity (fungi, plants, protists, animals)	1
ITS3	GCATCGATGAAGAACGCAGC	Broad range specificity (fungi, plants, protists, animals)	1
ITS4	TCCTCCGCTTATTGATATGC	Broad range specificity (fungi, plants, protists, animals)	1
ITS1-F	CTTGGTCATTAGAGGAAGTAA	Specific to fungi	2
ITS4-B	CAGGAGACTTGTACACGGTCCAG	Specific to basidiomycetes	2
NSA3	AAACTCTGTGCTGCTGGGATA	Specific to Dikaryomycota (primary forward primer)	3
NLC2	GAGCTGCATTCCCAACAACCTC	Specific to Dikaryomycota (primary reverse primer)	3
NSI1	GATTGAATGGCTTAGTGAGG	Specific to Dikaryomycota (secondary forward primer)	3
NLB4	GGATTCTCACCTCTATGAC	Specific to Dikaryomycota (secondary reverse primer)	3

¹ 1, White et al. 1990; 2, Gardes & Bruns 1993; 3, Martin & Rygielwicz 2005

(ascomycetes and basidiomycetes) was developed by Martin & Rygielwicz (2005) for use in a two-step PCR (nested PCR) where a larger fragment is amplified in a first PCR reaction and from this an internal fragment in a second PCR reaction. In the two-step PCR amplification of Dikaryomycota ITS sequences, primers NSA3/NLC2 serve as outer primers and NSI1/NLB4 as inner nested primers (Fig. 3, Table 1). Reamplification of an inner fragment of the first PCR product gives rise to greater sensitivity and allows to verify the identity of the product. However, although primers have been optimised for the amplification of fungal DNA specifically, such a specificity may ultimately cause a bias in the analysis. Therefore, primers have to be tested rigorously for the intended target range of species and eventually different primer combinations have to be used to prevent primer bias (Anderson et al. 2003).

DNA sequence databases

After amplification, the PCR fragments need to be analysed. The most detailed and specific information is obtained by sequencing. The ITS region can readily be sequenced as in most fungi it ranges from 600 to 800 bp allowing thus to obtain the complete forward and reverse nucleotide sequences with only two sequencing reactions. Once a sequence (of the ITS region) has been obtained for a sample in

question, it can be identified by comparing against other available sequences. The NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) or the EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk/embl/>) are public databases for all kinds of nucleotide (and protein) sequences and provide the highest number of entries for comparison. To find the most identical sequences, the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) is normally used directly on the database webpages. When working specifically with basidiomycete ITS sequences, the website of the Fungal Metagenomics Project at the University of Alaska can be used (<http://iab-devel.arcs.edu/metagenomics/>). It provides a FASTA search (for fast-all, a search algorithm providing a high level of sensitivity for similarity searching at high speed, Pearson & Lipman 1988) and a masking option which excludes highly conserved regions (SSU, 5.8S, LSU) from ITS similarity searches to force the search methods to find significant matches in the ITS 1 and ITS 2 regions (Geml et al. 2005). The specialised database is restricted to fungal ITS and LSU sequences compiled from NCBI GenBank and AFTOL (see below), making the search process faster and more specific. Further, it also provides an alignment option which helps in validating the obtained results. Nonetheless, the NCBI GenBank or the EMBL-Bank, as public databases open to sequence submission by anyone, have been shown to contain wrongly assigned sequences (Naumann et al. 2007). The amount of mistakes is actually very significant and it was estimated that upwards of 20% of the named sequences may be misidentified in some fungi (Bridge et al. 2003). To demonstrate this problem, we performed a BLAST search at the NCBI GenBank with the sequence of the ITS region of *Trametes versicolor* ATCC strain 32745. As shown in Fig. 4, the best hits (indicated by a low E-value) are in fact from *T. versicolor* strains. However, the next best hits then are from strains assigned to more distantly related species followed again by other strains assigned as *T. versicolor* and related *Trametes* strains, clearly indicating corrupt database entries.

With the Assembling the Fungal Tree of Life (AFTOL) project a database with molecular and non-molecular (e.g. morphological, ultrastructural and biochemical) characters is available to the scientific community which will be continuously updated as the understanding of phylogenetic relationships improves and additional annotations become available (Lutzoni et al. 2004, Celio et al. 2006, James et al. 2006, <http://ocid.nacse.org/research/aftol/>). The major advantage of this database is that its datasets are provided and verified by experts of fungal systematics and the type specimens for each taxa are carefully selected. Also, in the frame of the AFTOL project, computer programs have been developed to help deal with the ever-growing amount of sequence information. One program, named WASABI (for Web Accessible Sequence Analysis for Biological Inference), rates accuracy of newly submitted sequences in the AFTOL project, automatically assembles and compares them, and finally archives the manipulations and analyses performed (Pennisi 2005). Another program, dubbed more regularly (once a

week) mines the GenBank database for new LSU rDNA sequences of homobasidiomycetes, checks for redundancies, and performs automated phylogenetic analyses (Hibbett et al. 2005). With all these efforts, the AFTOL database should

Sequences producing significant alignments:		Score (Bits)	E Value
gi 40748278 gb AY504663.1	<i>Trametes versicolor</i> strain ATCC 32...	1172	0.0
gi 32264085 gb AY309018.1	<i>Trametes versicolor</i> strain ATCC 11...	1140	0.0
gi 32264083 gb AY309016.1	<i>Trametes versicolor</i> strain BCRC 36...	1124	0.0
gi 32264082 gb AY309015.1	<i>Trametes versicolor</i> strain BCRC 36...	1124	0.0
gi 32264084 gb AY309017.1	<i>Trametes versicolor</i> strain BCRC 36...	1118	0.0
gi 32264086 gb AY309019.1	<i>Trametes versicolor</i> strain BCRC 36...	1116	0.0
gi 13925666 gb AF261657.1	AF261657 <i>Tricholoma</i> sp. G1304 small...	1110	0.0
gi 21666964 gb AF455529.1	<i>Tricholoma robustum</i> isolate wb184...	1102	0.0
gi 58081983 dbj AB158314.1	<i>Trametes ochracea</i> genes for 18S r...	1102	0.0
gi 51039043 gb AY686706.1	<i>Trametes versicolor</i> strain KN9522...	1090	0.0
gi 10443877 gb AF251437.1	AF251437 <i>Phellinus igniarius</i> intern...	1086	0.0
gi 21666914 gb AF455480.1	<i>Tricholoma robustum</i> isolate wb316...	1080	0.0
gi 21666863 gb AF455434.1	<i>Phellinus igniarius</i> isolate wb434...	1070	0.0
gi 27085297 gb AY162175.1	<i>Laccaria fraterna</i> 18S ribosomal RN...	1055	0.0
gi 29465790 gb AY089738.1	<i>Hericiaceae</i> erinaceum isolate Fp-1024...	1031	0.0
gi 55783700 gb AY805633.1	<i>Trametes versicolor</i> isolate olin8...	995	0.0
gi 63024850 emb AJ699072.1	<i>Laccaria fraterna</i> ITS1, 5.8S rRNA ge...	991	0.0
gi 63024849 emb AJ699071.1	<i>Laccaria fraterna</i> ITS1, 5.8S rRNA ge...	991	0.0
gi 13136302 gb AF062634.1	AF062634 <i>Tricholoma robustum</i> CBS 494...	967	0.0
gi 49357483 gb AY636060.1	<i>Trametes versicolor</i> strain OE-241...	961	0.0
gi 15212247 gb AY046084.1	Basidiomycete sp. MUT 2750 18S rib...	944	0.0
gi 162632889 gb AY971627.1	Fungal sp. Sl.1.7 18S ribosomal RN...	902	0.0
gi 55801395 gb AY673076.1	<i>Coriolus versicolor</i> strain 471 int...	870	0.0
gi 134100076 gb AY354226.1	<i>Trametes versicolor</i> 18S ribosomal...	795	0.0
gi 1785799 emb J08749.1	PA3838588 unidentified basidiomycete 5.8	720	0.0
gi 21667025 gb AF461413.1	Basidiomycete isolate wb436 small...	712	0.0
gi 55818493 gb AY787683.1	<i>Trametes hirsuta</i> 18S ribosomal RNA...	700	0.0
gi 13325311 gb AF516556.1	<i>Trametes hirsuta</i> CultENN10198 SBI...	696	0.0
gi 62131842 gb AY972129.1	<i>Trametes hirsuta</i> strain ALP01 inte...	642	0.0
gi 6175576 gb AF139961.1	AF139961 <i>Irpex lacteus</i> 5.8S ribosoma...	640	1e-180
gi 13774456 gb AF347107.1	<i>Trametes versicolor</i> 5.8S ribosomal...	599	4e-168
gi 58081984 dbj AB158315.1	<i>Trametes maxima</i> genes for 18S rRN...	595	6e-167
gi 58081982 dbj AB158313.1	<i>Trametes hirsuta</i> genes for 18S rR...	595	6e-167
gi 14269072 gb AF363770.1	<i>Pycnoporus sanguineus</i> strain H2180...	587	1e-164
gi 14269065 gb AF363763.1	<i>Pycnoporus sanguineus</i> strain G53 1...	587	1e-164
gi 14269062 gb AF363760.1	<i>Pycnoporus coccineus</i> strain CBS 35...	587	1e-164
gi 14269061 gb AF363759.1	<i>Pycnoporus sanguineus</i> strain CBS 3...	587	1e-164
gi 14269060 gb AF363758.1	<i>Pycnoporus sanguineus</i> strain CBS 6...	587	1e-164
gi 14269057 gb AF363755.1	<i>Pycnoporus sanguineus</i> strain W006...	587	1e-164
gi 14269056 gb AF363754.1	<i>Pycnoporus sanguineus</i> strain W006...	587	1e-164
gi 14269073 gb AF363771.1	<i>Pycnoporus sanguineus</i> strain H2008...	583	2e-163
gi 14269071 gb AF363769.1	<i>Pycnoporus cinnabarinus</i> strain MUC...	581	9e-163
gi 14269055 gb AF363753.1	<i>Pycnoporus cinnabarinus</i> strain W30...	581	9e-163
gi 14269070 gb AF363768.1	<i>Pycnoporus cinnabarinus</i> strain MUC...	579	4e-162
gi 14269064 gb AF363762.1	<i>Pycnoporus sanguineus</i> strain G66 1...	579	4e-162
gi 14269063 gb AF363761.1	<i>Pycnoporus coccineus</i> strain CBS 35...	563	2e-157
gi 14269075 gb AF363773.1	<i>Pycnoporus sanguineus</i> strain G05 1...	561	8e-157
gi 14269069 gb AF363767.1	<i>Pycnoporus cinnabarinus</i> strain MUC...	541	8e-151
gi 33325318 gb AF516563.1	<i>Polyporus gramocephalus</i> CultENN11...	509	3e-141
gi 14269074 gb AF363772.1	<i>Pycnoporus cinnabarinus</i> strain I-9...	504	2e-139

Fig. 4 Result from a BLAST search at NCBI GenBank with the sequence from *Trametes versicolor* ATCC strain 32745. Entries labelled as *T. versicolor* (synonym *Coriolus versicolor*) are highlighted

become the most comprehensive and reliable tool for fungal species identification by sequence data for the scientific community. Whereas the reliability of the AFfOL database is provided, it can not cover all fungal species, thereby offering only a limited resolution for species assignments. In order to make the identification of wood decay fungi by sequences accessible to non-experts, a specialised database would be needed. Such a database would only include sequences from wood decay fungi, but with several individuals per species to cover the geographical and niche specific genetic variation. The database should certainly contain ITS sequences but, over time with more experience and accumulating knowledge, sequences from other indicative loci might also be included. Ideally, the sequences in the database would be backed by a fungal reference collection preserved in a herbarium or other institution. To overcome the problems caused by sequences from misidentified fungi in the GenBank, first specialist-backed databases have already been established and are continuously growing for selected groups of other fungi, i.e. FUSARIUM-ID (<http://fusarium.cbio.psu.edu>) to identify *Fusarium* isolates Geiser et al. 2004) and TRICHOBLAST (<http://www.isth.info/>) to identify *Trichoderma* and *Hypocrea* strains (Kopchinskiy et al. 2005), and UNITE (<http://unite.zbi.ee>) to identify ectomycorrhizal fungi (Kõljalg et al. 2005).

ITS fragment restriction analysis on agarose gels

Once the ITS sequences of a species is known, amplified ITS fragments from this species can be assigned by restriction digests (see also above). Compared to sequencing, this approach saves time and expenses but still yields the necessary specificity. An example of such an analysis is shown for the white rot *Schizophyllum commune* in Fig. 5. The sequence of the *S. commune* ITS fragments has been established before from various isolates (e.g. GenBank accession numbers AF249381, AF249386, AF249388) by mycological experts (James et al. 2001) and verified by us from mycelial and fruiting body reference samples included in the figure in lanes 4 and 10. ITS fragments amplified from *S. commune* DNA (primers ITS1/ITS4) were digested with the restriction enzyme *Hinf*I (DNA recognition sequence 5'-GANTC-3') that cuts them into three pieces of distinct size. DNA was extracted from decaying wood and outer bark samples, ITS fragments were amplified from the DNAs and also digested with *Hinf*I. The resulting patterns were then compared on an agarose gel with the patterns from the known samples. The gel electrophoresis shows that the DNAs from all wood or bark samples yield an identical pattern as the *S. commune* samples (fruiting body or mycelium). Therefore, we can conclude that *S. commune* is present in those samples. Two other fungi (*Coprinopsis xanthothrix*, *Trametes hirsuta*) were included in the study to demonstrate the differences between species.

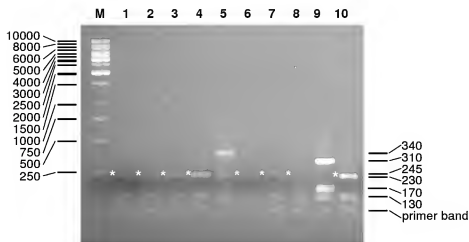


Fig. 5 PCR-RFLP of ITS1-5.8S rRNA-ITS2 region amplified from DNA extracted from wood, fungal fruiting bodies and mycelium. Fragments were amplified with primers ITS1 and ITS4 and digested with *Hinf*I. Lane M: 1kb DNA ladder, lanes 1-3: wood samples, lane 4: *Schizophyllum commune* fruiting body (from same wood samples), lane 5: *Coprinopsis xanthothrix* mycelium, lane 6-8: bark samples, lane 9: *Trametes hirsuta* mycelium, lane 10: *S. commune* mycelium. Band sizes are shown on the right, asterisks on the left of a lane mark bands with two fragments of nearly identical size

Conclusions

From all the available methods developed for the detection and identification of wood decay fungi in wood, PCR based methods of rDNA characterisation are ideal for many purposes. Due to the high copy number of the target genes and their amplification performed by PCR, a very high sensitivity is obtained. If combined with sequencing, species can accurately be identified. However, a reliable and more user friendly sequence database including only the relevant information is needed for the broad application. Other methods including spacial information, e.g. FTIR spectroscopy microscopy, will be useful for more specific questions, addressing the biology of the wood decay fungi and the mechanisms involved in the decay.

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10. Fourier Transform Infrared Microscopy in Wood Analysis

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Introduction

Deeper understanding in various areas of wood science and technology requires advanced analytical methods in order to define the morphological and chemical parameters of the structure of wood. Wood analysis often combines different types of analytical methods, for example carbohydrate and lignin content analysis, chemical analysis of extractives, nitrobenzene oxidation, ozonation, microscopy techniques, X-ray diffraction, NIR (near-infrared reflectance) spectroscopy, UV (ultra-violet) spectroscopy, advanced NMR (nuclear magnetic resonance) spectroscopy, thermogravimetry and others (e.g. see Donaldson et al. 1999, Brinkmann et

al. 2002, Bauch et al. 2004, Yeh et al. 2006). Traditional wet chemical analysis is useful to gain information on the average composition of wood samples. The minimal sample size required to meet the detection limit restricts the resolution to organ or tissue level and bulk samples, respectively. High spatial resolution is possible by light and electron microscopy. However, detection of chemicals in the sample usually requires dyes or labelling associated with problems like redistribution of the sample's chemical composition and unknown specificity or accessibility of binding sites (Blanchette et al. 1992, Srebotnik & Messner 1994, Vazquez-Cooz & Meyer 2002, Lang 2004, Drnovsek & Perdih 2005). In addition, only a single or a few selected compounds are detectable in a sample, which does not give an overview of the overall chemical composition.

The chemical processes during wood formation as well as during wood degradation by fungi and bacteria are too complex to be totally elucidated by wet chemical analysis and traditional microscopy. For applications in the wood industry, advanced analytical techniques are needed for identifying quality-determining factors of wood and wood products. Several techniques such as scanning or transmission electron microscopy with energy dispersive X-ray analysis (SEM- or TEM-EDX) and secondary ion mass spectrometry (SIMS) exist to visualise the elemental composition of the sample with high spatial resolution (Fritz 1989, Kuhn et al. 1997). A modern method especially suited to study the organic composition of wood with good spatial resolution is Fourier transform infrared (FTIR) imaging (Naumann et al. 2005, Naumann & Polle 2006). Imaging of the distribution of chemical compounds becomes possible by combining FTIR spectroscopy with microscopy (Salzer et al. 2000). FTIR spectroscopy is a well established method for chemical analysis of wood (Moore & Owen 2001).

The theory of infrared (IR) absorption

Above temperatures of absolute zero, i.e. -273.15°C , continuous vibrations exist in all atoms in a molecule with respect to each other (Sherman Hsu 2000, Günzler & Gremlich 2002). When the frequency of a specific vibration is equal to the frequency of the infrared (IR) radiation, molecules absorb the radiation. To calculate the possible number of vibrational modes of polyatomic molecules, the following has to be considered: Along the three Cartesian coordinate axes (x , y , z), any atom has three degrees of freedom corresponding to its motion. For a polyatomic molecule of n atoms, $3n$ total degrees of freedom exist. However, 3 degrees of freedom are required to describe the translational motion of the molecule (the net movement of an atom, ion or molecule through a gas, liquid or solid) and additional 3 degrees of freedom to describe the rotational motion of the entire molecule. Therefore, the true fundamental vibrations for a non-linear molecule will be $3n-6$. Only two degrees of freedom are sufficient to describe the rotation for linear molecules. Consequently, the fundamental modes of vibrations are $3n-5$. Those fun-

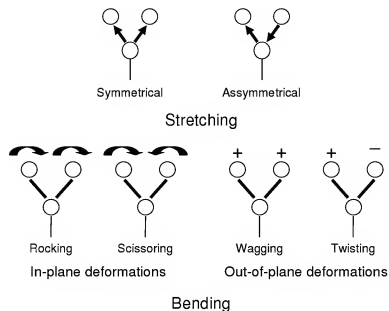


Fig. 1 Various vibrational modes for a non-linear group of a molecule. Arrows show the direction in which the atoms move during the vibration. + indicates motion from the plane of page towards and - away from the reader (after Christy et al. 2001)

damental vibrations that produce a net change in the dipole moment (product of either charge in an electric dipole and the distance separating them) are IR active. Molecules containing different types of atoms (e.g. SO_2 , CO_2 , etc.) can always interact with IR radiation, whilst diatomic molecules of one type of atoms (H_2 , Cl_2 , O_2 , etc.) cannot be excited to vibrate by lack of a dipole moment. The major types of molecular vibrations are stretching and bending vibrations (Fig. 1). Stretching vibrations are changes in the molecular bond length between two atoms and deformations are changes of the bond angle (Günzler & Gremlich 2002).

Generally in IR absorption spectra, the total number of observed absorption bands is different from the total number of fundamental vibrations because some modes of motion may be IR inactive and a single vibrational frequency may cause more than one mode of motion. Additional bands are generated by the appearance of overtones, combination of fundamental frequencies, differences of fundamental frequencies, coupling interactions of two fundamental absorption frequencies; and coupling interactions between fundamental vibrations and overtones (Sherman Hsu 2000).

There are a series of energy levels (vibrational energy states) associated with each of the vibrational motion of the molecule. By absorption of IR radiation, a molecule can proceed from one energy level E_{initial} to a higher energy level E_{final} . The difference in the energy levels ($E_{\text{final}} - E_{\text{initial}} = h\nu$) results in bending, stretching,

and twisting of the chemical bonds, leading to characteristic transmittance and reflectance patterns (Chalmers & Griffiths 2002, Günzler & Gremlich 2002).

FTIR analysis and microscopy – measurement principle

In FTIR analysis, a sample is subjected to IR radiation. Specific parts of the IR spectrum are absorbed by the sample according to its chemical composition (Günzler & Gremlich 2002). The stretch and deformation vibrations of specific molecular bonds in the sample, for example C-H, O-H, N-H, C=O, etc., result in absorption bands at specific wavenumbers of the FTIR spectrum. Wavenumber expressed in cm^{-1} is the traditional unit for FTIR analysis. Wavenumber is inversely related to wavelength as expressed by the following equation: **Wavenumber (cm^{-1}) = $10^7/\text{wavelength (nm)}$** . Wavenumber is proportional to frequency and thus to energy.

Irrespective of some exceptions, the IR spectrum can be split into four regions (Faust 1992):

- 4000-2500 cm^{-1} : the absorption of single bonds to hydrogen, e.g. C-H, O-H, and N-H
- 2500-2000 cm^{-1} : the absorption of triple bonds, e.g. $\text{C}\equiv\text{C}$ and $\text{C}\equiv\text{N}$
- 2000-1500 cm^{-1} : the absorption of double bonds, e.g. $\text{C}=\text{C}$ and $\text{C}=\text{O}$
- 1500-400 cm^{-1} : absorption owing to other bond deformations (complex interacting vibrations, e.g. rotating, scissoring, and some bending, see Fig. 1)

After transmission of the sample or reflection at its surface, an FTIR detector records the dispatched radiation signal, which can be transformed into transmission, reflection or absorption spectra. FTIR spectra are characteristic for the chemical composition of the particular samples as documented for poplar wood in Fig. 2, and bands can be tentatively assigned to specific molecular bonds or functional groups (Table 1). The FTIR spectra give a quick overview of the ratio of lignins, carbohydrates, proteins, lipids, aromatic and other compounds. Due to the complexity of biological samples and overlap of absorption bands, their assignment to specific molecular bonds or even to specific chemical compounds is not always unambiguous. A good reference library of spectra of pure components is helpful for band assignment. By comparison of complex sample spectra with reference spectra of individual components of the sample band assignments may be achieved. Tentative band assignments in FTIR spectra of wood components are available (e.g. Faix 1991, Pandey & Pitman 2003; see Table 1).

In addition to qualitative analysis, FTIR absorption spectra are suitable for semiquantitative or even quantitative analysis, because the proportionality of the absorbance (band area or height) to the concentration of a chemical substance follows the law of Lambert-Beer ($A = \lg I_0/I \approx \varepsilon cb$, A = light absorbance; I_0 =

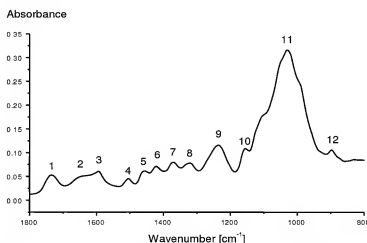


Fig. 2 FTIR ATR spectrum of powdered *Populus x canescens* wood (own analysis). For explanations of numbers at wavenumber maxima, see Table 1

radiant intensity incident upon absorbing layer, I = radiant intensity transmitted by absorbing layer, c = concentration of absorbing substance, ϵ = molar absorptivity, b = thickness of absorbing layer. In semiquantitative analysis, relative concentrations can be measured. For quantitative analysis, a calibration method with appropriate reference material has to be established.

An advantage of FTIR analysis is the possibility to analyse samples at very different levels of resolution. For example by powdering and analysing an aliquot of the powder, a representative spectrum of a wood block of any size can be obtained. In wood sections, with microscopy and a single channel detector, a spec-

Table 1 FTIR bands (wavenumber maxima are given) of powdered *Populus x canescens* wood and tentative assignments to wood components (according to Faix 1991, Pandey & Pitman 2003)

Wavenumber [cm ⁻¹]	Number in Fig. 2	Assignment
1735	1	C=O in xylans (hemicellulose)
1647	2	Absorbed O-H and conjugated C=O
1594/1505	3/4	Aromatic skeletal vibration in lignin
1455/1421	5/6	C-H deformation in lignin and carbohydrates
1371	7	C-H deformation in cellulose and hemicellulose
1319	8	C-H vibration in cellulose and C-O vibration in syringyl derivatives
1235	9	Syringyl ring and C-O stretch in lignin and xylan
1155	10	C-O-C vibration in cellulose and hemicellulose
1030	11	C-O vibration in cellulose and hemicellulose
897	12	C-H deformation in cellulose

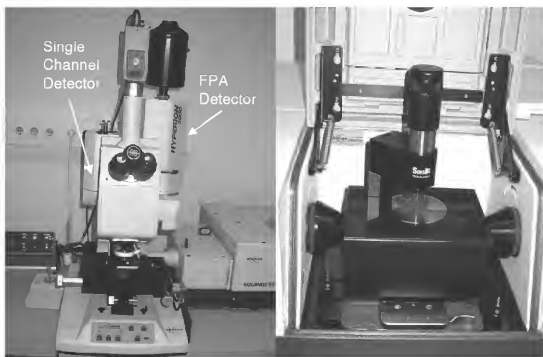


Fig. 3 The FTIR microscope Hyperion 3000 (Bruker Optics, Germany, Ettlingen) at the Institute of Forest Botany of the Georg-August-University Göttingen (left photo) is equipped with a single channel detector, a mid-infrared 64×64 mercury cadmium telluride FPA detector that can detect radiation in the wavenumber range between 3900 and 900 cm^{-1} , and a $15\times$ Cassegrain objective. The ATR unit with a diamond/zinc selenide ATR crystal in the FTIR spectrometer Equinox 55 (right photo) is installed in the box to the right of the FTIR microscope (photo left)

trum for a surface area of about $10 \times 10 \text{ }\mu\text{m}$ to $100 \times 100 \text{ }\mu\text{m}$ can be measured. However, the signal-to-noise ratio of FTIR spectra decreases with decreasing area of measurement. The highest spatial resolution on a relatively large sample area ($256 \times 256 \text{ }\mu\text{m}$) can be achieved with a focal plane array (FPA) detector measuring several individual spectra at a time in a spatially subdivided area (Salzer et al. 2000; see below). A microscope equipped with the two types of detectors is shown in Fig. 3.

Earlier, measurement of FTIR spectra required the preparation of KBr pellets by mixing KBr powder with sample powder and pressing these into transparent discs that subsequently were IR radiated (Günzler & Gremlich 2002). An easier and faster method to measure FTIR spectra of powdered wood samples or of plane surfaces of wood samples is to combine the FTIR spectrometer with an attenuated total reflection unit (ATR; Fig. 3). For analysis, the respective sample is pressed for intimate contact onto the ATR crystal (also called internal reflection element) that acts as a prism (for mode of function see Fig. 4). To obtain total

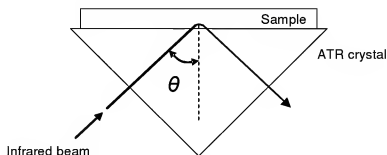


Fig. 4 Schematic representation of attenuated total reflection (modified from Günzler & Gremlich 2002). Angle of incidence θ

reflection at the interface between the sample and the ATR crystal, the infrared beam of the spectrometer is inserted into the ATR crystal at an angle exceeding the critical angle of incidence θ_c , which depends on the refractive indices of the ATR crystal n_1 and the sample n_2 according to the function: $\theta_c = \sin^{-1}(n_2/n_1)$. As a prerequisite for total reflection, the ATR crystal should have a higher refractive index (e.g. diamond/zinc selenide: 2.4) than the sample. At the interface between the ATR crystal and the sample, the infrared beam interacts with the sample and is attenuated by absorption according to the chemical composition of the sample.

By combining the spectrometer with a microscope (Fig. 3), the area of measurement can be visually controlled. A single channel detector measures one FTIR spectrum for a selected area, which can be sized with a rectangular knife edge aperture or a round pinhole aperture. By stepwise moving the sample under a single channel detector while taking a spectrum after each movement, so-called mappings can be recorded. The area of each band or the height of each point of each spectrum can be calculated by suitable computer software (e.g. Opus software by Bruker Optics, Ettlingen, Germany; <http://www.brukeroptics.com/opus/index.html>) and the semiquantitative distribution of the assigned molecular bonds or functional groups over the analysed sample area can be visualised as a grey scale or false colour mapping.

Mappings with single element detectors have been possible since decades, but they are very time consuming, taking several hours or even days depending on the overall mapping size, the sizes of the individual analysed areas, the spectral resolution and the number of repeats of measurements (scans). Since their invention in the nineteen nineties, FPA detectors have reduced the required measurement time to some minutes. These detectors consist of several thousands of single detector elements, which record all spectra at once without need for moving the sample (Salzer et al. 2000). The FPA detector in the FTIR microscope shown in Fig. 3 for example consists of 64 times 64 detector elements, each sized $4 \times 4 \mu\text{m}$, and thus simultaneously records 4096 spectra covering a total sample area of $256 \times 256 \mu\text{m}$. After integration of the whole dataset, it is possible to image the distribution of

the absorbance of each band or of a range of wavenumbers of all the spectra as a false colour image. The spatial resolution of such FTIR images is determined by the wavenumber of the radiation that can be processed by the FPA detector. A mid-infrared FPA detector for example may cover wavenumbers between 3900 and 900 cm^{-1} . At wavenumber 900 cm^{-1} , theoretically a maximum spatial resolution of 7 μm is possible (not considering the loss of resolution due to the numerical aperture of the objective). With increasing wavenumbers, the spatial resolution gradually increases. At wavenumber 1500 cm^{-1} and higher, a local resolution of up to 4 μm is achievable. The 4 x 4 μm size of the single detector elements defines this limit of resolution (Naumann & Polle 2006). Both types of spatially resolved FTIR measurements, i.e. mappings and imaging, are often referred to as micro-spectroscopy.

Applications of FTIR analysis

FTIR spectroscopy is a frequently used technique in various scientific and applied fields: e.g. material sciences (homogeneity of polymer films), natural history (age determination, success of conservation of natural materials), art (authenticity of paintings), forensic science (paint and fibre analysis), food technology (denaturation of meat), medical sciences (tumor detection), microbiology (identification of micro-organisms), and others (Snively & Koenig 1999, Gentner & Wentrup-Byrne 1999, Ngo-Thi et al. 2003, Kirschner et al. 2004, Lasch et al. 2004). In the field of wood science and wood technology, applications of FTIR spectroscopy emerged in identification of wood species (Barker & Owen 1999, Colom & Carillo 2005, Nuopponen et al. 2006a,b), in determination of the nature of wood modifications made for protection against biological threats and weathering (Zollfrank & Wegener 2002, Tingaut et al. 2005, 2006, Tjeerdsmma & Militz 2005), physical, chemical and microbial deterioration of wood including historical and modified wood (e.g. see Nuopponen et al. 2004, Pandey 2005, Genestar & Palou 2006, Pandey & Chandrashekar 2006, Prakash et al. 2006), sorption of chemical compounds by wood (Huang et al. 2006), fibre properties (Burgert et al. 2005), the nature of bonding in wood composites and wood-plastic composites (Lu et al. 2005, Marcovich et al. 2005, Okuda et al. 2006), and paper quality, aging and/or origin (Duran & Angelo 1998, Proniewicz et al. 1999, Jaaskelainen et al. 2003, Lojewska et al. 2005, 2006, Calvini et al. 2006, Eriksson et al. 2006, Polovka et al. 2006), and in analysis of lignin and wood pyrolysis and coalification (Sharma et al. 2004, Drobnjak & Masterlerz 2006, Fang et al. 2006).

In order to understand its chemical composition and structure, the main compounds of wood have in the past been intensively studied by FTIR spectroscopy: cellulose (e.g. Fengel & Ludwig 1991, Kataoka & Kondo 1998), lignin (e.g. Hergert 1971, Faix 1991), and hemicellulose (e.g. Marchessault 1962), both isolated and *in situ*. This information helps also in all the FTIR spectroscopy applications

in wood technology and paper and pulp industry listed above. Discrimination of softwood and hardwood species is easily possible, because of their differences in the guaiacyl-syringyl ratio (Faix 1991, Pandey 1999, Colom & Carillo 2005). Even closely related tree species can be correctly classified by FT-near IR analysis and soft independent modelling of class analogy (SIMCA) as demonstrated for different larch species (Gierlinger et al. 2004). Woods of the same species on different sites were shown to be discriminable by principal component analysis (PCA) of NIR spectra (Schimleck et al. 1996) or by simple cluster analysis of FTIR ATR spectra (Rana et al. 2007), which is important for identification of the origin of wood and its certification (further reading on wood certification in Chapter 8 of this book). Furthermore, cell-wall mutants can be recognised by FTIR spectroscopy - offering a way of screening for mutants with modified cell wall structure for research purposes and/or being better suited for applications in wood and paper industry (Chen et al. 1998; see Chapter 7 for further information on alterations of wood composition by genetic means). By increased formation of lignin and suberin, the genetic condition of resistance to the Dutch elm disease (mediated by the ascomycete *Ophiostoma novo-ulmi*) can be predicted in elms to a certain probability by FTIR spectroscopy (Martin et al. 2005). Chemical changes in wood caused through decay by white- and brown-rot fungi can be recorded by FTIR spectroscopy (Faix et al. 1991, Backa et al. 2001, Pandey & Pitman 2003, 2004, Mohebby 2005, Humar et al. 2006, Fackler et al. 2007) as well as effects of wood weathering by photo-irradiation and water leaching (Nuopponen et al. 2004, Pandey 2005). Such knowledge is of importance both for understanding the process of natural material recycling (see Chapter 17 of this book) and for undertaking effective measures in wood protection (see Chapter 13 of this book). To prevent wood decay, wood was treated by silylation or heat and the modifications were investigated by FTIR spectroscopy (Zollfrank & Wegener 2002, Tingaut et al. 2005, 2006, Tjeerdsma & Militz 2005).

In the academic field of wood research, the particular knowledge gained by FTIR analysis on the cell wall composition of non-woody plants can assist in the understanding of wood composition. The application of FTIR microspectrometry to primary plant cell walls for studying their heterogeneity and architecture was reviewed by McCann et al. (1997) and Dokken et al. (2005), including spectral fingerprinting of cell walls and their component molecules, characterising the structure and orientation of macromolecules, and functional chemical group mapping of a sample area. Defining the distinct differences of the complex cell walls of tracheids, vessels and fibres of woody plants requires however further investigation and necessitates comparative FTIR analysis of herbal and woody plants.

Until recently, only very few FTIR microspectroscopy studies on woody cell walls were available (Dokken et al. 2005). Most studies were carried out on the bulk sample level. For example, the chemical composition of *Pinus radiata* wood regarding total carbohydrates, Klason lignin, and extractives was calculated by par-

tial least square modelling of FTIR spectra (Meder et al. 1999). Mechanically and chemically isolated single wood fibres were chemically characterised (Burgert et al. 2005), and the hygroscopicity and the cellulose crystallinity of juvenile fresh cut pine wood and old pine wood used for over 200 years in roof rafters were determined (Esteban et al. 2006). FTIR microscopy has now also been documented to be well suited to investigate wood at the tissue level (Martín et al. 2005, Naumann et al. 2005, Naumann & Polle 2006, Peddireddi et al. 2006), the single cell and even the cell wall level in order to assess the variation between different cell types like ray parenchyma cells, latewood and early-wood tracheids (Hori & Sugiyama 2003). Moreover, mechanically and chemically isolated single wood fibres (Burgert et al. 2005) and the chemical composition from bark to pith of transgenic and non-transformed aspen were characterised by FTIR microscopy (Labbé et al. 2005). In combination with FPA detectors, such chemical information becomes available at a spatial resolution of 4–10 μm (see above), which allows the visualisation of the distribution of the chemical composition on the single cell wall level by false colour images (see front cover of the book). In the following sections, chemical imaging of wood and detection of fungi in wood by FTIR microscopy is described to emphasise this point.

Chemical imaging of wood by FTIR microscopy: lignin distribution

During sample preparation for chemical analysis in the FTIR microscope, care should be taken to avoid changes in the chemical composition of the sample. Since wood is structurally stable, embedding is in most cases not necessary. If samples are too soft or brittle for sectioning under fresh or dry conditions, cryo-sectioning is preferable to embedding in order to prevent changes in the chemical composition. For FTIR microscopy in transmission mode, the sample thickness has to be adequate. Samples have to be thin enough to allow sufficient transmission of the infrared radiation. From own practical experience, cross sections of a thickness about 10 μm or less are recommended for wood (Naumann et al. 2005).

Cellulose and lignin are the main compounds of wood (Eriksson et al. 1990, Morrell & Gartner 1998). Both have several absorption bands in the FTIR spectrum, which can be used for imaging their distribution in wood sections. The range from 1530 to 1490 cm^{-1} is tentatively assignable to lignin (Faix 1991; Table 1). By integrating this range, an image of the lignin distribution in wood sections can be calculated (Naumann & Polle 2006). To demonstrate the lignin distribution in *Populus x canescens* (see Fig. 5 and front cover of this book), a 10 μm thick section of 3-month old poplar wood was analysed with an FTIR microscope Hyperion 3000 in conjunction with an FPA detector (Fig. 3).

The light microscopic view of the analysed poplar wood section (Fig. 5, upper panel, left and front cover of this book) enables exact orientation in the corres-

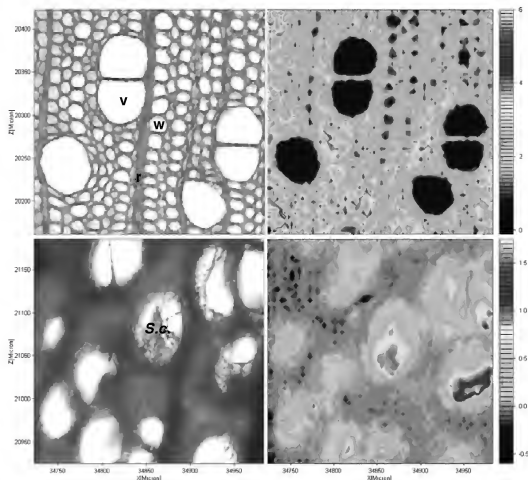


Fig. 5 Fourier transform infrared (FTIR) microscopy of poplar wood and beech wood infected with *Schizophyllum commune* (for a colour version of the same figure, see front cover of this book). Sections were measured on a KBr window (2 mm thickness) as an infrared transmissive sample holder. FTIR spectra were recorded for the wavenumber range of 3900 to 900 cm^{-1} with a spectral resolution of 12 cm^{-1} . Sixteen spectra were co-added and averaged to improve the signal-to-noise ratio. Evaluation of FTIR data and calculation of the FPA false colour images (right upper and bottom picture) was performed by the Bruker Optics OPUS version 5.0 software (<http://www.brukeroptics.com/opus/index.html>). Top panel: Uninfected poplar wood (v = vessels, w = wood fibres, r = xylem rays) as seen in the light microscope (left) and false colour image of the same wood section (right) measured with an FPA detector of the FTIR microscope Hyperion 3000 (Fig. 3) using the range between 1530–1490 cm^{-1} to estimate the lignin distribution. Bottom panel: *S. commune* (S.c.) infected beech wood as seen in the light microscope focussed on fungal hyphae (left) and false colour image of the same section measured with the FPA detector of the FTIR microscope illustrating the distribution of the mycelium within the sample (right). The image was calculated by correlating a typical part of the spectrum (1190–940 cm^{-1}) taken from mycelium (see also Fig. 6) within vessels with the 4096 spectra of the full FPA data set.

ponding false colour FPA image (Fig. 5, upper panel, right and front cover of this book). Vessels (v), wood fibres (w) and xylem rays (r) are well discernable. In the coloured FPA image on the front page of the book, blue represents no lignin, while green and red corresponds to middle and high lignin content, respectively. Cell walls in the FPA image appear broader than in the light microscopic view (see Fig. 5) because of the lower resolution of FTIR microscopy. The cell walls of poplar shown in the figure have a thickness of about 1 to 2 μm . As the frequency of infrared radiation and the pixel size of the FPA detector limit the spatial resolution to 4 μm (see above), the cell walls measured by just one single detector element of the FPA detector appear as being 4 μm thick. A further limitation to be considered for quantification is that the adjacent empty lumina also contribute to the signal intensity and, therefore, lead to an underestimation of chemical components at the intersection of wall to lumen (Naumann & Polle 2006). In conclusion, these physical limitations result in spatial blurring of objects of smaller dimensions than those of the detector units and are the reason that single cell walls can not be further resolved to differentiate individual cell wall layers.

Detection of fungi in wood

FTIR spectroscopy has successfully been used to identify various bacteria and also some fungal species. Even different strains within a species can be distinguished (Naumann et al. 1991, Mariey et al. 2001, Ngo-Thi et al. 2003). Fungal studies addressed first species with relevance to medicine and food industry, such as *Candida albicans*, *Saccharomyces cerevisiae*, and various edible and toxic mushrooms (Naumann et al. 1991, Ngo-Thi et al. 2003, Liu et al. 2004). More recently, spectra of a number of white-rot fungi (Naumann et al. 2005, Peddireddi et al. 2006, A. Naumann, unpublished data; Fig. 6) as well as of mycorrhizal species have been established (A. Naumann & A. Polle, unpublished results), with the task of species as well as strain identification.

Early detection of fungi in trees, dead wood in nature or in service are of great scientific and economic interest, as described in Chapter 9 of this book. Spectral differences between wood and fungi can be used to detect a general presence of fungi inside wood, but they also provide a potential to identify particular fungal species within the wood. Mycelium from the two white-rot fungi *Schizophyllum commune* and *Trametes versicolor* were clearly distinguished by FTIR analysis within as well as on beech wood. Spectra for mycelium of the same fungus growing within and on the beech wood differed considerably from each other, indicating an influence of the overall environmental conditions on the fungal spectra (Naumann et al. 2005). Nutritional conditions such as different wood species and age of a mycelium have been shown to be factors that influence the spectra (S. Peddireddi, unpublished).

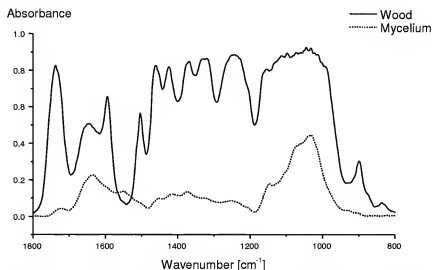


Fig. 6 FTIR spectra of wood fibres and mycelium of *S. commune* measured in beech wood sections with an MCT single channel detector of an FTIR microscope. For sample preparation, cross sections (30 μm) of infected beech wood blocks were cut with a sledge-microtome and air-dried flatly under cover slips with a weight on the top. For the measurements of selected areas with wood fibres and mycelium, respectively, an aperture of 20 x 45 μm and a spectral resolution of 4 cm^{-1} were used. 30 scans were co-added and averaged (Naumann et al. 2005)

As an example, the imaging of the distribution of white-rot fungus *S. commune* in beech wood 4 month after infection is presented in Fig. 5 (lower panel) and, in colour, on the front cover of the book. In the light microscopic view of the beech wood section, fungal mycelium of *S. commune* (marked *S.c.* in the figures) is visible in several vessels. Using the trace computation function of the OPUS software (Bruker Optics, Ettlingen, Germany; <http://www.brukeroptics.com/opus/index.html>) to calculate the distribution of the fungal mycelium within the beech wood section, a characteristic part of the spectrum obtained from mycelium in vessels (1190-940 cm^{-1} ; see also Fig. 6) was correlated with the 4096 spectra of the FPA data set taken from the whole beech wood section (Fig. 5, lower panel at the right). In the corresponding false colour image shown on the front cover of the book, yellow and red zones correlate to areas of dense fungal mycelium and indicate medium and high mycelial content within the beech wood vessels.

Conclusions and perspectives

FTIR spectroscopy is an established technique for determining the chemical composition of various biological and chemical samples. The technique finds more and more important applications, for example in quality control of industrial products, including products of the wood industry. In basic research, FTIR spectros-

copy contributes to understand the biological processes of wood production and wood degradation, and also of chemical processes of natural environmental as well as technically forced wood modifications.

In combination with modern FPA detectors, the chemical composition of wood at the tissue and cell level can be spatially studied by FTIR microscopy. The frequency of infrared radiation and the pixel size of the FPA detector limit the spatial resolution to 4 μm . Unequal distributions of cellulose, lignin, hemicellulose, protein content and composition are detectable over a study area without *a priori* knowledge which changes might occur. FTIR analysis is not restricted to pure wood, but can also be used to detect and study the chemical composition of fungi living within wood and the effects they have on the chemical structure of the wood. FTIR results on first collections of mycorrhizal and wood-decay fungi promise that species identification of micro-organisms interacting with living trees and with dead wood will become possible with this technique. Another interesting area of FTIR application is seen in the identification of wood species, wood genotypes, and wood origin.

Currently in microspectroscopy, we make use of characteristic bands and specific small areas in the wood and fungal spectra (Rana et al. 2007; A. Naumann, unpublished). In order to utilise the much more informative entire spectral information of the large FPA data set, multivariate statistical analysis is necessary. The aim is to group spectra with common features into classes to reduce the large amount of data into more meaningful and interpretable data sets (Salzer et al. 2000, Lasch et al. 2006, Heraud et al. 2007). Such data sets will allow additional discrimination.

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11. Volatile Organic Compounds for Wood Assessment

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Introduction

Utilisation of **volatile organic compounds (VOCs)** for the quality assessment of wood is basically a bionic concept which is inspired by the impressive achievements of insects in performing this task just by olfaction in order to exploit wood as a resource. The word **“bionics”** is made up of the two words “biology” and “electronics”. In German, however, the second part comes from “Technik”, which means engineering in this context. In English, this approach of combining biology and engineering is often also called **“biomimetics”**. The two expressions are used more or less synonymously. The interdisciplinary field of bionics is about scrutinising and transferring “natural inventions” into technical applications. In the course of evolution, nature has developed, improved and tested these inven-

tions over millions of years. For technical exploitation, the optimised solutions to a specific set of problems have firstly to be thoroughly analysed. Subsequently, the newly described solutions can be implemented in technical applications with corresponding boundary conditions.

In order to highlight possibilities and limits of an assessment of wood by detecting VOCs released by wood, the first part of this contribution deals with the genesis of VOCs in wood, in fungi and in wood infested by fungi. In the second part, the interaction with insects is used as example how nature exploits the content of information encoded in patterns of VOCs released by wood. By examination of the recognition processes of insects and a parallel trace analysis of related VOC patterns released by fungus infested wood, we might learn how to assess wood quality as well as the kind and state of fungal infestation by detecting wood-released VOCs in a quick and non-destructive manner. In view of that, current techniques are displayed enabling the detection of specific VOCs or of patterns of VOCs released by wood, in order to suggest possible lines of development for devices assessing wood quality.

Volatiles released by living trees

In the discussion of greenhouse gases and their impact on global climate changes (see Chapters 5 and 6 of this book), there is an increasing interest in the complex chemistry of the troposphere. The dynamics of the global atmospheric chemistry through climate forcing is triggered by VOCs (Holopainen 2004, Dindorf et al. 2005). Beside VOCs of anthropogenic origin, especially VOCs emissions from forests which are covering ca. 30% of landmass (FAO 2006) are sources affecting the system. The quantities of **volatiles of natural origin (NVOC)** released above the main landmasses as arable land and forests exceed by far the quantities from anthropogenic sources. Due to their dominance, reactivity and physical properties, they are classified as **VVOCs (very volatile organic compounds like methane), reactive VOCs** (isoprene and terpenes) and **non-reactive VOCs** (Guenther et al. 1995).

VOC-emissions by plants are unavoidable due to their metabolic activities (Peñuelas & Llusà 2004). A dominant reactive VOC released by forests for example is isoprene, which is widespread but not generally present throughout the plant kingdom (Harley et al. 1999, Owen & Peñuelas 2005). Isoprene is discussed to play an important role in tropospheric chemistry (Fehsenfeld et al. 1992, Lerdau et al. 1997). Similar to terpenes, its reactivity influences the atmospheric dynamics of ozone, formation and deposition of organic nitrates and organic acids (Harley et al. 1999). Due to this importance in atmospheric processes, algorithms were developed describing the dependence of isoprene and terpene emissions of plants on light and temperature (Dindorf et al. 2005). Further factors as drought, diurnal and seasonal variation or growth conditions were discussed as parameters influen-

cing the VOC emissions of plants (Dudt & Shure 1994, Staudt et al. 2001, 2003). However, there are undisputable many additional internal (e.g. genetic, biochemical) and external (e.g. interaction with fungi and insects) factors that affect the presence (Litvak & Monson 1998) and emission of different VOCs by trees and other plants (Apel et al. 1999, Peñuelas & Llusà 2001, Schütz et al. 2004) which are not yet covered by known algorithms.

Most trees are grouped, due to their affinity, in coniferous and broadleaved species. This is also reflected in their VOCs composition: VOCs differ highly from coniferous to broadleaved woodlands. Regarding coniferous trees, VOC-research is almost exclusively done in the family of Pinaceae, e.g. *Pinus*, *Picea*, *Larix*, *Abies*, *Tsuga*, and *Cedrus* (Hayward et al. 2004, Lee et al. 2005). Broadleaved species were examined on a somewhat broader scale comprising Fagales (*Betula*, *Fagus*, *Quercus*), Sapindales (*Acer*, *Castanea*) and e.g. Salicaceae (*Salix*, *Populus*) (Pasteels & Rowellrahier 1992, Tollsten & Müller 1996, Hakola et al. 2001, Paczkovska et al. 2006). Further genera such as *Eucalyptus* (Guenther et al. 1993, Zini et al. 2002) are characterised and several comparative studies screened plant species for single VOCs only (Owen et al. 1997). Plant VOCs are mostly alkanes/alkenes, aromatic hydrocarbons, alcohols, phenolics, terpenes, esters, aldehydes and ketones (Kesselmeier & Staudt 1999). However, due to technical restrictions, the analytical window covers currently only compounds with boiling points between 60°C to 250°C at atmospheric pressure, and of intermediate to high thermal stability (Schütz 2001).

General processes in plant cells, as the lipoxygenase (LOX)-pathway (Feussner & Wasternack 2002) are responsible for the release of generalistic VOCs as the so called **“green leaf volatiles” (GLV)**. Mainly alcohols, aldehydes of linear six carbon chains and their derivatives such as (Z)-3-hexen-1-ol, (Z)-3-hexen-1-yl-acetate, hexan-1-ol, and (E)-2-hexenal belong to this group (Visser 1979). Whereas the name GLV implies the paradigm that only leaves (not needles) are releasing these compounds, it was proven that coniferous trees release these compounds, too, but only in minute amounts (Schütz et al. 2004). GLV are released in low rates from nearly every plant species (Hatanaka 1993) and show a typical increase on mechanical wounding (de Bruxelles & Roberts 2001, Mithöfer et al. 2005) of any type of plant tissue, be it leaves, needles, stems or roots (Matsui 2006). Especially young developing leaves and damaged leaves - and leaves are damaged by wind or insects in a forest all the time - release increased rates of GLV. With regard to the function of trace compounds with low emission rates as **carrier of information (“info-chemicals”)**, these minor components must however not be neglected (Schütz 2001, Schütz et al. 2004). GLV are known to play an important role in insect attraction and aggregation (Visser 1979, Schütz et al. 1997, 2004, Ruthr 2000) or insect repellence (Huber & Borden 2001, Zhang & Schlyter 2004) and even in signalling between plant individuals, known as the phenomenon of **“talking trees”** (Tschamtko et al. 2001, Arimura et al. 2002, Engelberth et al. 2004,

Farag et al. 2005). All this points out a complex interactive defence system in plants in which the VOCs play the role of a language. VOCs carry information about the constitutive or induced defence status of the plant, whether it is mechanically wounded, attacked by insects or micro-organisms (Schütz et al. 1997, Schütz 2001, Holopainen 2004, Weissbecker et al. 2004, Holighaus & Schütz 2006, Johné et al. 2006a,b, Paczkowska et al. 2006).

Isoprenoids are characteristic defence chemicals of conifers and are produced through the mevalonate (MEV) or methyl-erythritol-diphosphate (MEP) pathways (Keeling & Bohlmann 2006). They are highly variable in structure (>30,000 terpenes are known) and occur in trees as isoprene (C₅), monoterpenes (C₁₀), sesquiterpenes (C₁₅) and diterpenes (C₂₀) (Sharkey & Singaas 1995, Phillips & Croteau 1999, Trapp & Croteau 2001). Following just the name of a compound, for instance, α -pinene should not be mistaken in that it is exclusively released by coniferous trees like *Pinus* spp.. For example, European beech (*Fagus sylvatica*, Fagaceae) seems to be a much stronger monoterpene emitter than expected. The monoterpenes of this species, studied by Dindorf et al. (2005) and Moukhtar et al. (2005), are dominated by sabinene with more than 90% of the daily terpene emission, but the typical coniferous volatiles α -pinene and β -pinene were also found in the VOC pattern of beech trees. This holds also true for *Quercus suber*, the cork oak (Pio et al. 2005). α -pinene, sabinene, β -pinene and limonene were the main compounds (80%) among the released terpene fraction from the oak.

Within taxonomic groups of lower plants, the VOC patterns are more alike, based on a more similar biochemistry of secondary plant compounds (Asakawa 2004). This relationship is treated in the scientific field of chemotaxonomy (Harborne & Turner 1984). However, variability of VOC patterns can be high, notwithstanding the degree of relationship. The Southern beech *Nothofagus dombeyi* releases α -pinene in considerable amounts, whereas five other species of *Nothofagus* do not at all (Quiroz et al. 1999). A similar variability was shown by Harley et al. (1999) for isoprene emission of several woody and herbaceous plant species of Northern America.

Volatiles released by trunks and deadwood

Trees provide a huge variety of plant tissues and plant surfaces. Compared to herbaceous plants, their surface is much bigger and more sculptured resulting in a higher variety of local VOC pattern and subsequent niches for interacting organisms. For example, 80% of VOCs stored in and released by needles of *Pinus* sp. and *Picea* sp. are identical with those released by the trunk of the trees, but they display significantly different quantitative patterns of VOCs (Sjödén et al. 2000, Schütz et al. 2004). In contrast to leaves and needles, there are only few systematic examinations about the influence of internal or external parameters on VOC patterns or released VOC quantities of wood or bark of trees (Schütz et al. 2004, Ho-

lighaus & Schütz 2006). However, various commercial interests lead to the analysis of chemical bark contents. Bark (root or trunk) as well as wood (root or trunk) are outstanding sources for commercial products since rich in essential oils (Wang et al. 2005) which are often VOCs. These defence chemicals against attacking organisms often display antibiotic activity and are used for various aspects in human life, e.g. in medicine (Kalemba & Kunicka 2003), food (Burt 2004) and personal care products (Priest 2002). Besides, VOCs are examined for applications in biotechnical plant protection and biotechnical stored product protection (Manker 2005).

At the beginning of the dying process of a tree, a remarkable differentiation of the ecological system "tree" takes place resulting in a tremendous diversity of species of insects and micro-organisms (Harmon 1986, Moore et al. 2004). The exact point of initiation of the dying process, whether caused by storm, insects, fungi or other circumstances, is often hard to define. Although felling or breaking down is often stated as the borderline between living tree and deadwood, when looking closely to physiological and chemical processes, a clear separation is hardly possible. Continuously during life, cells of healthy trees die and are rebuilt. Programmed cell death (PCD) is an integral part of plant development and also of defence. It occurs at all stages of the life cycle, from fertilisation of the ovule up to death of the whole plant. Indeed, without it, tall trees would probably not exist (van Doorn & Woltering 2005). Permanent stress of the environment like oxidative stress, heat or draught, infestation by micro-organisms, etc. causes the loss of protective compounds which have to be renewed (Sharkey & Singaas 1995, Blokhina et al. 2003, Loreto et al. 2006 and citations therein). The oxidation of unsaturated fatty acids as constituents of lipid membranes or storage compounds of cells leads to the production of aliphatic aldehydes, alcohols, alkanes and other VOCs (Feussner & Wasternack 2002). These kinds of compounds can all the time be found produced in bark and wood (Weissbecker et al. 2004, Holighaus & Schütz 2006). Attacks of fungi and insects increase oxidative stress on the plant tissue and, in the course, the emission rates of these VOCs (de Bruxelles & Roberts 2001, Schütz 2001). Such biotic stress occurs very often in living plants and in many instances it can be overcome or healed. If the plant is however not able to cope with the related damage, the dying process is initiated. Regardless of whether biologically initiated or caused by felling, the end of a tree does not result in "chemical silence" since not all the cells of a tree are at the time dead. The defence system and other cell functions are still working, until the storage pools are empty and dying is completed. In case of such deadwood, the decay process of a tree and wooden substrate results in an extensive release of VOCs, changing considerably due to abiotic environmental factors (humidity, temperature etc.) and biotic interactions (fungi, micro-organisms and insects) (Paiva 2000). The complexity of decay is demonstrated by the VOCs released from bark of a *F. sylvatica* trunk subsequently to felling (Fig. 1, 2). Felled beech trunks release more than 140 volatile



Fig. 1 Distribution of physiological decay states on beech trunk; grey to black patches: fresh to seriously decayed (modified from Holighaus & Schütz 2006)

compounds in detectable amounts during the first phase of decay (0–2 years after felling), up to 70 of them simultaneously. Differences between small bark samples hint at a high spatial variability of chemical processes of decay and related volatiles within one trunk (Fig. 1; Holighaus & Schütz 2006).

Starting with felling, the number of volatiles and the emission rates of aldehydes increase (Holighaus & Schütz 2006). Following the decay progress, exemplary chromatograms yielding from a gas-chromatographic separation and subsequent mass-spectrometric detection of VOCs released by the bark of a beech trunk are displayed in Fig. 2 with the main compounds named. Several simple and branched alcohols occur at the beginning of the fermentation process in the headspace of bark tissue (Fig. 2B). Beside terpenes, phenolic compounds as 2-methoxy-phenole, 4-methoxy-phenole and 1,2-dimethoxy-benzene emanate during the phase of oxidising bark tissue. They vanish fast and the branched alcohols change to longer straight-chained alcohols (Fig. 2B, C). At initial infestation with white rot fungi, up to 30 sesquiterpenes are additionally detected in the bark samples (Fig. 2C). After predominant degradation of lignin and cell structures of the bark by fungi, only sesquiterpenes are left to release (Fig. 2D).

VOCs emitted by wood and wood products

Wood is one of the most widespread building materials. For usage in constructions, the fading of natural metabolic processes in wood is enhanced by drying. The dried “deadwood” does not any more release VOCs on the basis of metabolic processes of the wood cells and, also, a part of the constitutive defence VOCs evaporated during the drying processes. VOC release rates differ between different drying and modification processes (Otwell et al. 2000). Air dried wood releases 8 times more VOCs than thermally modified wood (Manninen et al. 2002). The thermal modification has a high impact on wood chemistry and constructive properties. Thermally modified wood is dominated by aldehydes (hexanal), carboxylic acids and -esters, air dried coniferous wood by terpenes (Tjeerdsmas et al. 1998).

Analytical research on VOCs released by wood and wood products is performed by two reasons. Firstly, several VOCs released from wood are suspected

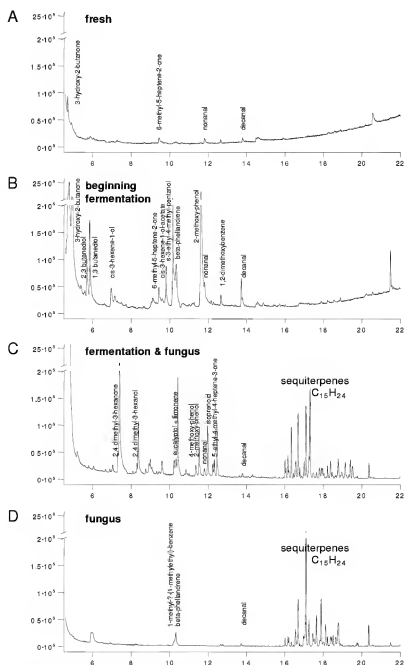


Fig. 2 VOC patterns released by differently decayed bark patches on a trunk of European beech (data from Holighaus & Schütz 2006)

to be toxic or cancerogenic to human beings. The main focus of examinations is therefore on toxic VOCs as well as on unpleasant odours (Bleich et al. 1998). Compared to solid wood, the release rate of VOCs of several derived timber products is significantly reduced (e.g. OSB=0.25x, MDF=0.05x), whereas the rate of

aldehyde emission is much higher (Barry & Corneau 1999, Risholm-Sundman et al. 1998, Risholm-Sundman 2002). Of high concern are toxic formaldehyde emissions of processed wooden products (Sundin & Roffael 1992, Bleich et al. 1998, Schäfer & Roffael 2000; see Chapters 15 and 16 of this book). Glues and binding agents are releasers of this compound (Chapter 15 and 16 of this book). According to Marutzky & Roffael (1977) and own examinations, freshly cut wood itself releases considerable amounts of formaldehyde surpassing sometimes even legal thresholds. However, the quantity of formaldehyde emissions of cut wood decreases quickly. Usually, after 6 month of storage, formaldehyde emissions of wood are below detection limits. Other natural compounds from wood discussed in the context of toxicity belong to the group of monoterpenes (Johansson 1999, Jentoft & Stray 2002). However, the positive affection to wooden products is strongly influenced by the perception of a typical wood-odour and needs also to be considered.

The second focus on VOCs of wood material involves several indoor moulds and fungi using the wooden substrate for growth, thereby generating additional VOCs being of considerable concern regarding the “sick-building-syndrome” (Mølhave et al. 1997, Johansson 1999, Fischer & Dott, 2003, Wilkins et al. 2003, Portnoy et al. 2005; see below and Chapter 12 of this book).

Volatiles released by fungi

Fungi are organisms that obtain nutrition by out-of-body digestion, releasing a range of extracellular enzymes to digest their substrates. For degrading wood, they produce cellulases (endo- and exo-cellulases), hemicellulases, α -glucosidase and oxidase, phenoloxidases and laccases (Eaton & Hale 1993; see Chapters 17 and 19 of this book). They further utilise the generated decomposition products for processing metabolism, extending mycelium, and in some cases, developing their fruiting bodies (Kües 2000; see also Chapter 22 of this book). Besides obtaining energy and nutrients, metabolic activity yields also volatile by-products including VOCs. This attributes to the typical odour of each fungus. For example, the edible champignon, oyster mushroom, shiitake, puffball, truffle and straw mushroom all have their own individual aromas (Mau et al. 1997, Venkateshwarlu et al. 1999, Mauriello et al. 2004, Zawirska-Wojtasiak 2004, Chiron & Michelot 2005), motivating our appetite. What contributes to these emblematic odours or VOCs? What is their purpose and function? There is still much about the fungal metabolism, especially the secondary metabolism, to uncover.

Classes of VOCs released by fungi

VOCs released from wood and wood-decaying fungi range from low to high molecular weight and can be further sub-divided by their chemical structure (Korpi et al. 1998, Gao & Martin 2002, Schleibinger et al. 2005, Chiron & Michelot 2005,

Table 1 VOC classes as released by wood, infested wood, and micro-organisms (data taken from Korpi et al. 1998, Gao & Martin 2002, Schleibinger et al. 2005, Chiron & Michelot 2005, Gao et al. 2005, Thakeow et al. 2006)

Chemical categories	Examples
Alcohols	Ethanol, isopropyl alcohol, octan-1-ol, octan-3-ol, 1-octen-3-ol
Aldehydes	Acetaldehyde, benzaldehyde, furfural, nonanal
Acids	Acetic acid, methyl butanoic acids, 2-methyl propanoic acid
Ketones	Acetone, pyranones, hexanones, heptanones, octan-3-one
Esters	Ethyl acetate, methyl propanoate
S-containing compounds	Dimethyl disulfide, dimethyl trisulfide
N-containing compounds	Methyl pyrimidine, pyrazine, cyclobutyl amine
Isoprenoids:	
Monoterpenes	α -pinene, β -myrcene, 3-carene, limonene
Oxidised monoterpenes	Borneol
Sesquiterpenes	Farnesenes, barbatenes, protoilludenes
Oxidised sesquiterpenes	Longiverbenone

Gao et al. 2005, Thakeow et al. 2006) into the eight broad categories of alcohols, aldehydes, ketones, acids, esters, S- and N-containing compounds and isoprenoids (monoterpenes, oxidised-monoterpenes, sesquiterpenes and oxidised-sesquiterpenes) as listed in Table 1.

The un-branched C8 compounds, 1-octen-3-ol, octan-3-one and octan-3-ol, are considered to be typical fungal constituents found in such diverse species as *Aspergillus*, *Fusarium* and *Penicillium* strains (Schnürer et al. 2002), *Tuber borchii*, *Tuber mesentericum*, *Tuber excavatum* (Mauriello et al. 2004, Menotta et al. 2004), *Lentinus* sp., *Agaricus bisporus*, *Agaricus campestris*, *Lactarius* sp., and *Calvatia* sp. (Overton 1994), and wild *Polyporus sulfureus* and *Fistulina hepatica* (Wu et al. 2005a,b). However, not only filamentous fungi emit these C8 compounds, but, to a lesser extent, yeasts and bacteria, too (Bruce et al. 2004, Nilsson et al. 2004, Schleibinger et al. 2005). On beech wood, the wood rotting fungi *Trametes versicolor*, *Poria placenta* and *Gloeophyllum trabeum* all released the isoprenoides α -pinene, 3-carene, longifolene, and cedrene in addition to the alcohols 1-octen-3-ol and octan-3-ol, and the ketone octan-3-one. In addition, each fungus had its own characteristic compounds in the sesquiterpene-class, *T. versicolor* for example α - and β -barbatene, *G. trabeum* high amounts of protoillud-6-ene, and *P. placenta* fair amounts of daucene (Thakeow et al. 2006).

Impact of fungal development on VOCs released by fungi

VOCs released by fungi can change considerably during their life cycles. For instance, the VOCs produced by live and dead mycelium of *Serpula lacrymans* grown on *Pinus sylvestris* shavings were found to be different. Living mycelium released 1-

Table 2 VOCs released from *T. borchii* fruiting bodies during ascus maturation [ascus stage 0: 0-5%, 1: 6-30%, 2: 31-70%, and 3: 71-90% of the sets of spores in the asci are mature, respectively (after Zeppa et al. 2004)]

VOC-Class	Ascus stage			
	0	1	2	3
Isoprenes	<ul style="list-style-type: none"> • 2-methyl-5-(1,2,2-trimethylcyclopentyl)-, (S)-phenol • valencene • α-patchoulene • longiverbenone • cedrene • aromadendrene 	<ul style="list-style-type: none"> • longifolene • β-cedrene • borneol 	<ul style="list-style-type: none"> • isopinocamphe • 3-thujene 	<ul style="list-style-type: none"> • D-limonene • <i>trans</i>-ocimene • (R)-α-pinene • α-farnesene • 4-isopropyl-tropolene
N-containing compounds	<ul style="list-style-type: none"> • 3-(1-piperazinyl)propanamide 			
S-containing compounds			<ul style="list-style-type: none"> • 5-methyl-3H-pyran-1,2-dithiol-3-one • 2-(1,1-dimethylthioxy)-5-methylthiophene 	<ul style="list-style-type: none"> • 3-methyl-thiophene • 2,3-dihydro-5-methylthiophene • 1-(methylthio)-1,3-butadiene • 2-methyl 4,5-dihydro-thiophene • 5-methyl-3H-pyran-1,2-dithiol-3-one • 5,6-dihydro-2H-thiopyran • ethyl <i>tert</i>-butyl sulphoxide

octen-3-ol as a major volatile component, and dead mycelium 3-methylbutanal and 2-methylbutanal, but only trace amounts of 1-octen-3-ol (Ewen et al. 2004).

The change of VOCs in sexual development was followed in fruiting bodies of the ascomycete *T. borchii* over four different stages of spore maturation which were defined by the percentage of asci containing mature spores (Zeppa et al. 2004; Table 2). The stages differed in number and type of VOCs. Immature asci and asci at the end of sporulation released sesquiterpenes, whereas S-containing compounds are released only at the later stages of ascus development. Interestingly, the sesquiterpene aromadendrene released by the immature ascus was also found produced by *T. borchii* mycelium grown in the presence of its host plant

Tilia platyphyllos but not by free-living mycelium (Zeppa et al. 2004). In conclusion, vegetative and reproductive stages of fungal development produce different sets and also quantities of VOCs, likely as a result of the activation of different metabolic pathways. Therefore, VOC patterns can be used as a destruction-free probe system in order to explore biochemical processes underlying developmental processes of the fungi.

Impact of substrate on VOCs released by fungi

Growth and development of fungi are strongly dependent on nutrients and the physical environment (Kües 2000, Chang & Miles 2004), although they can adapt to a broad scale of conditions. Changes in growth conditions influence their metabolisms, resulting in altering VOC patterns (Wheatley et al. 1997, Gao & Martin 2002). For example during spirits production with carbohydrate-rich substrates such as potato or wheat, the yeast *Saccharomyces cerevisiae* produces ethanol as main product, but the individual substrate provides different and characteristic aroma, caused by the minor components of the yeast and also the substrate (Conner et al. 1998, Pinheiro et al. 2001, Kafkas et al. 2006, Porto et al. 2006).

Some investigations have been carried out on the impact of different media on VOC patterns released by micro-organisms (Wheatley et al. 1997, Bruce et al. 2000, Fiedler et al. 2001, Gao & Martin 2002, Gao et al. 2002, Scotter et al. 2005). Using two main groups of amino acid-rich and carbohydrate-rich media for microbial growth, it was put forward that there are VOCs unique to bacteria and fungi which are called **unique microbial volatile organic compounds (UMVOCs)** (Gao & Martin 2002). VOCs released from fungi on carbohydrate-rich media are mainly alcohols, acids, aldehydes and ketones. In case of amino acid-rich media, higher quantities of nitrogen (N)- and sulphur (S)-containing VOCs are encountered, for instance, cyclobutylamine and dimethyl trisulphide, respectively (Bruce et al. 2004). Zygomycetes, ascomycetes, and deuteromycetes are likely to release the S-containing compound methanethiol when propagating on protein-rich media, in contrast to basidiomycetes (Scotter et al. 2005; Table 3). In comparison, bacteria on protein-rich media release also broad ranges of VOCs, most markedly S-containing VOCs like dimethyl trisulfide and heptan-2-one, the latter one independently of the media (Gao & Martin 2002).

Looking closer at moulds, substrates have a strong effect on VOC production by different species. When *Aspergillus* spp. grow on media rich in nutrients, they proceed the normal primary metabolism and release in course alcohols like 3-methyl-1-butanol, 2-methyl-1-propanol, 1-octen-3-ol and ketones like octan-3-one. Once the nutrients are exhausted, the fungi shift to special secondary metabolisms which yields changed VOC patterns. More and other VOCs are released, including terpineol from the terpene group. In case media are amino acid-rich, this leads to production of S-containing VOCs (Fiedler et al. 2001, Gao & Martin 2002). Moreover, some aspergilli can accept sulphur from inorganic substrate and release it in

Table 3 Low molecular weight VOCs released from different types of fungi grown on C- and N-rich media, respectively (data from Scotter et al. 2005)

Phylum	Zygomycete		Ascomycete		Deuteromycete		Basidiomycete	
Species	<i>Mucor racemosus</i>		<i>Apergillus</i> spp.		<i>Fusarium solani</i>		<i>Cryptococcus neoformans</i>	
Media	C-rich	N-rich	C-rich	N-rich	C-rich	N-rich	C-rich	N-rich
VOCs								
Ethanol	+	+	+	+	+	+	+	+
Acetaldehyde	-	+	-	+	+	-	+	+
Acetone	-	+	-	+	-	-	+	-
Methanethiol	-	+	-	+	-	-	-	-

+ found, - not found

form of dimethyl-disulfide (Gao & Martin 2002). The situation is contrasting in *Stachybotrys chartarum*, which releases about five times higher quantities of VOCs, when exploiting rich media. VOCs released by *S. chartarum* belong to the group of alcohols, ketones and terpenes. Also *Trichoderma* spp. (*Trichoderma pseudokoningii*, *Trichoderma viride*, *Trichoderma harzianum*) release different VOCs when grown on rich malt extract and poor minimal media, respectively (Wheatley et al. 1997, Fiedler et al. 2001, Humphris et al. 2001; see Chapter 14 of this book for potential biological functions of VOCs of *T. viride*). However, no N-containing VOCs are observed, very low amounts of S-containing VOCs (benzothiazole) are released in *T. viride*, and ethanol is produced in large amounts, independently of the substrate types.

Most wood-rotting fungi belonging to the basidiomycetes on artificial nutrient-rich medium as well as on the natural substrate wood typically release linear aliphatic C8 compounds such as 1-octene, octan-1-ol, 1-octen-3-ol, 2-octenal, 2-octen-1-ol, octan-3-one, and octan-3-ol (Rösecke et al. 2000, Ewen et al. 2004).

These examples from the literature document that VOCs released by micro-organisms are certainly useful to distinguish different groups and even species, but environmental and physiological conditions have to be considered. In order to gain a more consistent picture about growth conditions (temperature, humidity and light), kind of media and developmental stages have to be clearly and in depth addressed in research, since these factors strongly affect the metabolism, leading consequently to changes in VOC patterns (Table 4).

Volatiles released by fungus-infested wood

As discussed already above, VOCs released from fungi are strongly dependent on substrates and the stage of the life cycle and development. Wood as a substrate for wood-decaying fungi can be expected to influence the fungal metabolism. Fungi attack and colonise wood in different ways, depending on the properties of the

Table 4 VOCs released by micro-organisms (data taken from Wheatley et al. 1997, Bruce et al. 2000, 2004, Fiedler et al. 2001, Gao & Martin 2002, Gao et al. 2002, Scotter et al. 2005)

Micro-organisms	Bacteria		Zygo- mycetes		Asco- mycetes		Deutero- mycetes		Basidio- mycetes	
Nutrients (C/N rich)	C	N	C	N	C	N	C	N	C	N
VOC categories										
Alcohols										
• C1-C5	+	+	+	+	+	+	+	+	+	+
• C6-C10					+	+			+	
Aldehydes										
• C1-C5				+		+		+	+	+
• C6-C10		+				+			+	
Ketones										
• C1-C5	+	+			+	+		+	+	
• C6-C10	+	+				+			+	
• C10+	+	+								
Acids										
• C1-C5	+									
Esters										
• C1-C5	+									
Alkanes/Alkenes										
• C1-C5	+				+	+				
• C6-C10						+	+			
S-containing										
• C1-C5	+			+		+				
N-containing										
• C1-C5		+								
• C6-C10	+									
Terpenes										
• monoterpenes	+				+		+		+	
• sesquiterpenes					+				+	

wood. Wood as a substrate can be living or dead - namely in form of a standing tree, a felled tree, storage wood and wood in service, respectively -, engaging more or fewer living cells in the wood with the ability to render the substrate. When standing in the forest, the tree is a suitable nutrition source for fungi since it contains nutrients like sugars, amino acids and minerals. Anyway, when fungi infect a living tree, they have to adapt to or protect themselves against the tree defense system. In dead wood in contrast, most defence systems of the tree are not anymore active. However, dead wood provides less free sugar and amino acids, and more polymerised substrate which is more difficult to digest. Therefore, when

fungi are growing on living or dead wood, the fungal metabolism and, thus, the fungal VOC patterns will be differentially affected. Besides nutrient contents, the water content of the wood is also severely affecting fungal growth and development, why in European standard EN 335-1 (1992) the hazard class for fungal decay is related to the water content in wood.

Most fungi that infest and decay wood belong to the phylum of basidiomycetes, more precisely to the class of homobasidiomycetes. These wood-decaying fungi are divided into two main types, brown- and white-rot fungi, respectively, according to the colour of the wood in an advanced stage of decay. This difference results from their ability to degrade lignin. Brown rot fungi can degrade all components in wood but lignin. The left-over phenolic substrate lignin turns brown in colour. In contrast, white rot fungi can degrade all types of wood components, even lignin. Their decay mechanism resembles the action of a bleaching agent resulting in whitish-stained cellulose as left-over from the wood (see also Chapter 17 of this book). Another type of fungi softens the cell walls of wood by decay reactions. Such species are therefore called soft-rot fungi and they belong mostly to the phylum of ascomycetes. So far however, little is known between the effect of different rotting abilities of fungi and the release of respective VOC patterns (Thakeow et al. 2006).

Sick building syndrome (SBS) as a consequence of VOCs

When micro-organisms infest buildings, they may produce a potentially hazardous environment. Individuals exposed to environments that contain high concentrations of airborne contaminants from microbial organisms report health symptoms including eye and sinus irritation, headache, nausea, fatigue, congestion, sore throat, and even toxic poisoning. The term “sick-building syndrome” (SBS) was first coined in the mid-1980s referring to ill-health symptoms arising from poor indoor air quality, that further on have been frequently correlated with the presence of fungi (Ahearn 1996). Current methods for detecting microbial contamination include air and material sampling with fungal culture analysis, air sampling coupled with gas chromatography-mass spectrometry, and visual inspection (Pasanen 1992, Schiffman et al. 2001). Several micro-organisms infest buildings and release **microbial volatile organic compounds (MVOCs)**. Typical fungi are of the genera *Aspergillus*/*Euotium*, *Penicillium*, *Cladosporium*, *Trichoderma* and *Stachybotris*. MVOCs released are mainly alcohols (pentanols, hexanols, octanols), ketones (hexanones, heptanones, octanones), and a few N- and S-containing compounds (pyrazine and dimethylsulfide, respectively) (Wilkins et al. 2003, Nilsson et al. 2004, Schleibinger et al. 2005).

Besides contaminants released by micro-organisms, wooden buildings themselves also release VOCs which contribute to SBS. VOCs released from several wood species were examined (Risholm-Sundman et al. 1998), i.e., ash, beech,

maple, birch, oak, cherry, rubber wood, pine and spruce. Acetic acid, a compound of corrosive nature, was emitted from every wood species, except pine and spruce. In contrast, terpenes are generally released from pine and spruce wood. Especially 3-carene may irritate skin and mucous membranes. Allergy and chronic lung function impairment might be elicited after prolonged exposure (Falk et al. 1991).

What is the role of VOCs for insects?

Wood is the basis of existence for adapted fungi and insects, influencing each other's living conditions. In the context of the trophic interaction between wood, insects, and fungi, we have seen the functions of the participants and the variation within. Wood can be living or dead, actively or passively defensive. It can be infested by specific insects and/or fungi – under indoor and outdoor environments. Insects can follow just wood VOCs (Weissbecker et al. 2004), fungal VOCs (Fäldt et al. 1999, Holighaus & Schütz 2006) or defense signals (Schütz & Weißbecker 2003). On the one hand, this broad and diverse information is a basis for diverse evolutionary development, marking ecological partitioned niches and suitable environments for hosting species such as insects. On the other hand, this requires a high plasticity of appendant organisms as receivers of the available chemical information (Johné et al. 2003, 2006a). Hence, relying on common VOCs keeps flexibility in a changing and dynamic environment whereas relying on a specific VOC as a kind of marker compound for suitable host plants represents the advantage of highly specific adaptation. Thus, research on a multitrophic system using VOCs as information needs advanced techniques in trace analysis and interpretation (Weissbecker et al. 2004). Sometimes, the crucial information is small and silent, maybe hidden behind abundant noises.

VOCs mediating insect interaction with trees, wood and fungi

Insects on living trees

Insects attacking living trees use the typical host VOCs released by the tissue sought after. In stems of conifers, for example, several monoterpenes such as α -pinene, β -myrcene, terpinolene and β -pinene are attractive to a large number of conifer inhabiting beetles: an overview of the chemical ecology of bark beetles (Scolytidae) in this complex olfactory landscape is given by Byers (2004), of weevils (Curculionidae) by Schlyter (2004), of longhorn beetles (Cerambycidae) by Allison et al. (2004), and of jewel beetles (Buprestidae) by Schütz et al. (1999a, 2004). The influence of VOCs on insect behaviour is well studied in the case of *Picea abies* in the context of infestation with the bark beetle *Ips typographus*. A cascade of VOCs is released during the process of infestation and colonisation by the beetle: primary attractive VOCs from the bark draw beetles to a weakened tree, followed by production and release of aggregation pheromones by the insects. Subsequent-

ly, the release of VOCs from the tree indicating exhaustive overuse of the plant resource leads to repulsion and dispersal of the beetles (Byers 2004). The prospect of successful infestation led obviously during evolution to a high sensitivity of tree invading insects to VOC signals related to different stress factors. Defence reactions of the tree become transparent through shifts in VOC abundance and composition (Pettersson & Boland 2003, Schütz et al. 2004). Franceschi et al. (2005) reviewed defence aspects by the wood anatomy influencing chemical defences against insects and blue-staining fungi. Both, anatomical and chemical defense turn out to be strongly interlinked (Hudgins et al. 2004, Erbilgin et al. 2006, Zeneli et al. 2006).

Fungus-insect interaction on trees, trunks and deadwood

Fungi often participate in tree-insect-interactions. These interactions with trees and wood are reviewed by various authors in the past (e.g. Buchner 1953, Wilding et al. 1989, Vega & Blackwell 2005). Insects can be a vector of fungi (Paine et al. 1997), feed on the fungi degrading wood (Mueller et al. 2005), or even host endosymbiotic fungi for wood digestion (Buchner 1953). Especially many xylophagous insects feeding on deadwood co-evolved with fungi to complex symbiotic coenosis (Douglas 1989, Klepzig et al. 1996, Dillon & Dillon 2004). Enzymatic detoxification abilities of these endosymbiotic fungi make otherwise protected lignocellulosic resources accessible - not at least hence, these fungi and their enzymes are of commercial interest (Dowd 1992). Conversely, because of competition for the same resource volatiles from wood decaying fungi can be repellent for insects (Johné et al. 2006a) or toxic fungal metabolites (VOCs and non-VOCs) may keep insects away from the wood (Seybold et al. 2006). Overall, even healthy trees are not aseptic. Fungal interactions with living trees are known in forms of latent infections of the xylem or endophytic colonisations of leaves (Hendry et al. 2002), not to mention the symbiotic mycorrhiza (van der Heijden & Sanders 2002). In the xylem of European beech, for example, *Hypoxylon fragiforme* was identified as a latent invader besides other casual inhabitants (Hendry et al. 2002). Chemotaxonomic studies of this species give hints to metabolites released by the fungus (Stadler et al. 2004). However, up to now there are no data about chemical reactions, resultant VOCs, or insect preferences emerging from this type of fungus-tree interaction.

The insects related to tree trunks can be grouped roughly into phloem- and xylem-feeders. Phloem is rich in nutrients but strongly shielded by the active plant defense system and xylem is hard to digest but less protected (Lieutier 2004). Fungi can play a fundamental role for both groups of insects to overcome the respective defensive systems (Dowd 1992). Moreover, insects may feed on fungi utilising the ability of fungi to catabolise cellulose (Watanabe & Tokuda 2001). Some xylophagous or deadwood insects are therefore grouped as mycetophagous insects, too (Bouget et al. 2005). For example, the family of bark beetles (Scolytidae) with

worldwide about 6000 species presents a huge variability of associations with trees and fungi (Jacobs & Wingfield 2001, Kolařík et al. 2005). These beetles differ widely in their ecology and biochemical adaptations to their host trees. Within this taxonomic group are phloem- and xylem feeders, ambrosia beetles with a compulsive association to symbiotic fungi and there are also several facultative connections between bark inhabiting insects and fungi (Farrell et al. 2001, Aukema et al. 2005, Mueller et al. 2005). The majority of Scolytidae are phloem-feeders with obviously mutualistic relationships to their fungal associates but the strength of interaction is still subject of considerable debate. Several cases are known where insects act as vectors of serious fungal pests, most noticeable when non-indigenous, newly introduced, and thus not adapted to a given environment (Harrington et al. 2001, Allen & Humble 2002). Many fundamental aspects of the degree of dependence in such insect-fungus relationships are however still poorly known (Kirisits 2004). A strong relationship to fungi is known in the scolytid xylophagous ambrosia beetle *Trypodendron domesticum* and the lymexylid *Hylecoetus dermestoides* which both infest the xylem of *F. sylvatica* trees. They follow the first chemical hints of weakness in living and especially freshly cut trees and initiate ongoing decay processes by introducing several associated "ambrosia" fungi (Farrell et al. 2001, Holighaus & Schütz 2006). VOCs are the main signals for these beetles obtaining information about precise decay and defence status of trunk patches (Holighaus & Schütz 2006). Electrophysiological techniques (EAG = electroantennography) use insect antennae, which are often much more sensitive to VOCs than trace analytical methods, to locate within the hundreds of VOCs those, carrying the information of suitability. General and omnipresent VOCs are little informative. Others correlate with general plant physiological processes and are therefore useful for an assessment of suitability of a trunk as breeding substrate. Further VOCs give highly specific information of e.g. certain fungal species colonising the wooden substrate which can be indispensable for insect development and hence lead to attraction (Belmain et al. 2002), or which are even fatal and have to be avoided. Evolution eventually led to highly specific adaptations which turned VOCs into triggers of these complex interactions. By observing these sensitive signals with analytical techniques, we can obtain the state of wood in aging, decaying and the status of interaction or infestation with fungi and insects (Weissbecker et al. 2004, Holighaus & Schütz 2006, Thakeow et al. 2006).

Insects on wood and wooden products

Not all insects feeding on wooden substrate necessarily need fungal associates. There are species without as *Ergates faber* (Cerambycidae), producing own endogenous cellulases. This ability for cellulose degradation is found sometimes in other insect families, too, for example in cockroaches (Blattaria) and termites (Isoptera) (Douglas 1989, Watanabe & Tokuda 2001). Beside "wood worms"

which are larvae from the family of Anobiidae, the old house borer *Hylotrupes bajulus* (Cerambycidae) is a widespread insect pest of coniferous timbers in buildings. Without any fungal support it can cause substantial damage to roof timbering or framework houses even in temperate climate. An understanding of the volatiles relevant for the orientation of *H. bajulus* could help to find new methods for protection of wood and a control of the beetle (Reddy et al. 2005a). *H. bajulus* is very delicate in the choice of sites for mating and oviposition and obviously, it is guided by olfactory cues. Recent behavioural studies assigned importance to monoterpenoid hydrocarbons as attractants (Fettköther et al. 2000, Reddy et al. 2005b). A direct investigation of the olfactory response of *H. bajulus* to original odour samples of its host trees by GC-EAD/MS yielded a more complex mixture of terpenes, aldehydes, alcohols and other hydrocarbons as VOCs being important to *H. bajulus* (Weissbecker et al. 2004). This knowledge will be crucial for the assessment of thermal wood treatments or chemical wood modification techniques for protecting constructional wood without any poisonous chemicals, just by reducing olfactory traceability and attraction for the old house borer.

Siricid woodwasps (Siricidae) (Thomsen & Koch 1999) and Anobiidae, like the death watch beetle *Xestobium rufohilosum* (Belmain et al. 2002), are xylophagous insects which have acquired fungal associates and cause substantial damage on wood and wooden products prior and during service. Both are examples for endosymbiotic relationships to fungi. Ambrosia beetles (Holighaus & Schütz 2006), as described above, are known for ectosymbiotic relationships. In termite-species (Isoptera), (Brune & Friedrich 2000) both types of symbiosis can be found.

Insects on fungi

Other beetles, for instance of the family of Cisiidae, do not bother to prepare the wood for symbiotic fungi but just feed in more or less specialised manner on fruiting bodies of bracket fungi (Jonsell & Nordlander 2004). *Fomitopsis pinicola* and *Fomes fomentarius*, bracket fungi growing on tree trunks of *Pinus* and *Fagus* species, respectively, were shown to release C8 compounds, such as 1-octene, octan-1-ol, octan-3-ol, 2-octene-1-ol, and 1-octen-3-ol, and sesquiterpenes such as β -barbatene. The cisid beetles *Cis glabratus* and *Cis quadridens* can discriminate the host odour of fruiting bodies of *F. pinicola* and *F. fomentarius*, respectively (Fäldt et al. 1999). Moreover, predatory Anaspidae feeding on cisid beetles, namely *Anaspis marginicollis*, *Anaspis rufilabris* and *Epinotia tedella*, were significantly attracted to 1-octen-3-ol released predominantly by damaged fruiting bodies of the bracket fungi (Fäldt et al. 1999). These different degrees of specialisation in insects for fungus infested wood might be used in biomimetic sensor systems for the assessment of wood with respect to fungal infestation.

Techniques for assessing wood quality on the basis of VOCs

It is a bionic concept to utilise VOCs as a parameter for wood quality assessment. This concept is inspired by the impressive achievements of insects in performing this task just by olfaction and taste. However, the approaches to copy these "inventions of nature" are so far sparse.

Biosensors

The selectivity and sensitivity of biological recognition processes motivated the development of biosensors. Biosensors are miniature measuring devices consisting of a biological recognition component in close spacial and functional contact with a physical transducer unit. The biocomponent utilised can be of different levels of organisation: whole (micro-)organisms in organismic biosensors, whole sensory systems in biosensors on the basis of sensory organs, and enzymes, antibodies, or receptor-proteins as well as nucleic acids in bio-molecular biosensors. The physical transduction unit is needed to transduce analyte caused changes of the biocomponent (heat, mass, light, resistance, capacity, current, potential, ...) into output signals that can be processed by electronic data processing units (Wollenberger et al. 2000; see also Chapter 12 of this book).

Biosensors for detecting volatile compounds principally fight the problem that biocomponents tend to deteriorate when exposed to air. Especially biomolecules have to be protected by membranes which are hindering diffusion of VOCs and thus, compromising sensitivity. Hence, most biosensors utilising biomolecules as a biocomponent are relying on extraction of VOCs by aqueous solvents, as it is done for the amperometric enzyme biosensor for detecting phenolic compounds from wood pulp (Rosatto et al. 2001), and the amperometric enzyme biosensor for the assessment of wood ageing (Campanella et al. 2005).

In contrast, biosensors on the basis of immobilised micro-organisms show considerable working stability in air. One major field for these biosensors is the measurement of complex parameters like toxicity. Biosensors for the toxicity of VOCs using recombinant bioluminescent *Escherichia coli* bacteria (Gil et al. 2000, Mwinyihija et al. 2005) or different strains of algae (Podola et al. 2004) were designed, which might be applicable for wood quality assessments with regard to potential health implications of wood materials. Moreover, biosensors on the basis of immobilised micro-organisms were proposed for the analysis of fermentation characteristics of spoilage micro-organisms (Wang & Wang 2002). This proposal was picked up utilising a potentiometric biosensor based on immobilised yeast cells (Rotariu et al. 2004), and for the analysis of microbial communities utilising an array of electrochemical microsensors and microscale biosensors selectively responding to volatile fatty acids based on immobilised bacteria (Meyer et al. 2002,

Revsbech 2005). These biosensors might be applicable for wood quality assessments with regard to microbial infestation and degradation.

Biosensors on the basis of insect olfaction provide unrivalled measuring rates, selectivity and sensitivity in the analysis of VOCs in air (Schroth et al. 1999). Integrating ecological and behavioural observations with electrophysiological measurements of antennal responses to VOC mixtures corresponding to specific situations (for instance: forest fires) yielded sets of insect antennae and marker compounds for the detection of these situations. The black jewel beetle (*Melanophila acuminata*) was found to be attracted by forest fires, because burnt wood is the only suitable substrate for bringing up its offspring. The antennae of the black jewel beetle were proved to be highly sensitive and selective to guaiacol derivatives, compounds which are generated by the pyrolysis of wood (Schütz et al. 1999a). The antennae and with them the set of detected compounds can thus be appointed by the biosensor-designer to assess wood species and fire parameters (temperature, oxygen access) involved in the forest fire in distances of kilometres (Schütz 2004). A few of the compounds recorded by the beetle's antennae are recognised to be of special interest to coopers' thermally modified oak wood used to produce barrels for wine storage (Chatonnet et al. 1999, Campbell et al. 2005), identifying a promising field of application for such a biosensor in process and quality control of thermally modified wood. Thermal and chemical wood modification is recently discussed to replace treatments of construction wood with poisonous biocides (see Chapter 13 of this book). The house borer *H. bajulus* is one of the major reasons for this problematic biocide treatment (see above). Besides terpenes, there are aldehydes involved in detection and classification of wood by the beetle (Weissbecker et al. 2004). A biosensor on the basis of the antennae of *H. bajulus* can be useful to assess the VOC patterns generated by different wood modification techniques with regard to the detectability and acceptability of treated wood by this dangerous beetle.

The detection and characterisation of fungal infestation and decay in construction wood prior to and during service is another important task by the fact that fungal infestation can have deleterious impacts on the mechanical stability of construction wood. According to their ecology and behaviour, different insect antennae respond differentially to microbial VOCs. However, virtually every insect examined responds to branched- and linear C-8 compounds as markers for microbial activity (Fäldt et al. 1999, Schütz & Weißbecker 2003, Holighaus & Schütz 2006). With this knowledge, biosensors on the basis of insect antennae for the infestation by micro-organisms were established (Schütz et al. 1999b). Despite the high performance of this kind of biosensors, their field of application is limited to on-site-measurements because of the limited life-time of the biocomponent antennae, ranging from hours to days. Further development of biomimetic sensors employing key principles of stabilisation, pre-filtering and recognition of insect olfaction

will be necessary in order to extend life time and availability of sensor devices on the basis of insect olfaction (Schütz et al. 2001).

Electronic noses

The biological olfactory system inspired furthermore the development of electronic nose technology. An electronic nose is a machine that is designed to detect and discriminate complex mixtures of VOCs (odours) using a sensor array (Eberheim et al. 2004). The sensor array consists of broadly tuned (non-specific) sensors that are treated with a variety of odour-sensitive biological or chemical materials. An odour stimulus generates a characteristic fingerprint from the sensor array. Patterns or fingerprints from known odours are used to construct a database and train a pattern recognition system so that odours within the trained range can subsequently be classified and identified. Thus, electronic nose instruments are comprised of hardware components to collect and transport odours to the sensor array as well as electronic devices to digitise and store the sensor responses for signal processing (Pearce et al. 2003).

The pulp and paper industries in eastern Canada need to differentiate black spruce, balsam fir, and jack pine because their proportions in wood chips affect the quality of the pulp and paper produced. A prerequisite to determine their proportions is to be able to rapidly identify the wood of the three conifers. Using a combination of marker compounds and GC profiles of hexane extracts made a distinction even of the sapwood of these tree species possible (Pichette et al. 1998). However, this initial method is too slow and expensive to be used by paper mills. A more advanced electronic nose consisting of 32 conducting polymer sensors (Cyranose™ 320) was able to rapidly discriminate and identify black spruce, balsam fir and jack pine wood chips, utilising principal component analysis as a data analysis tool (Hobbs et al. 2000, Garneau et al., 2004). In another application, thermally modified oak wood for wine barrels was assessed regarding its toasting level by an electronic nose consisting of 6 metal oxide semiconductor sensors utilising principal component analysis, discriminant function analysis and neuronal network techniques for data analysis (Chatonnet 1999).

The growth of bacteria and fungi on organic matter generates a broad range of VOCs (see above). Most studies with electronic noses deal with the detection and classification of bacteria (Holmberg 1997, Gardner et al. 1998), using sensor-arrays consisting of six to nine metal oxide semiconductor gas sensors. Few reports are available for fungal detection: with an accuracy of 93% six spoilage fungi of meat (four *Eurotium* spp., each one *Penicillium* and *Wallemia* species) were classified on blood agar 24 hours after infestation and prior to visible growth, using an electronic nose consisting of 14 polymer sensors (Keshri et al. 1998). With the same electronic nose, seven homobasidiomycetes (*Agaricus arvensis*, *A. bisporus*, *A. campestris*, *Agaricus maleolens*, *Agaricus nivescens*, *Pleurotus sajor-caju*, and *Volvariella*

bombycina) were differentiated (Keshri et al. 2003). Twenty-four hours after their inoculation on rich potato-dextrose-agar (PDA) and a minimal medium (Czapek-Dox agar), 5 fungi suspected to be involved in SBS (*Aspergillus flavus*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*, and *S. chartarum*) were detected and classified by an electronic nose designed at North Carolina State University (NC State E-Nose) consisting of 15 metal oxide sensors. The classification was independent on type of the growth medium. The raw data of this analysis were transferred to an electronic data processing system. They were first compressed using windowing functions which provided a set of four features for each sensor. Linear-discriminant analysis was then applied to the compressed data to maximise class separability. Sixty percent of the compressed data were randomly selected to form a training set for the classification algorithms. K-nearest-neighbours (KNN) and least-squares (LS) techniques were both employed to classify the remaining 40% of the compressed data. The KNN technique resulted in 90% accuracy of species identification after the first day of inoculation (Schiffman et al. 2000).

Outlook

Bionic noses integrate two different approaches by copying algorithms used by nature in odour perception on different level of organisation. Biosensors selectively tuned to marker compounds are amended by algorithms of electronic noses operating with an array of broadly tuned chemical sensors in order to discriminate complex situations based on a set of marker compounds.

As a complex sensing device, an insect antenna can serve as a blueprint for technical sensor optimisation in a “constructive bionics” approach, using for instance the principles of the porous cuticle for sample enrichment, as shelter against air and dust, and as a chemical pre-filter. Algorithms used by insects for contrast enhancement in odour mixture recognition can be exploited as a source of inspiration in an “informational bionics” approach. The identification of marker compounds or pattern recognition algorithms from sensory ecology of insects interacting with wood and degrading micro-organisms might serve as a guideline in a “process bionics” approach in the development of new bionic sensors for wood assessment. Thus, in the near future the possibilities in wood assessment can be considerably extended by thorough application of a bionics/biomimetic approach.

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12. Biological Monitoring of Pollution in Forests and of Pollution caused by Wood Utilisation

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Introduction

Our environment is exposed to various kinds of pollutants from anthropogenic origin such as urbanisation and human activities in agriculture and industry. Volatile, soluble and solid hazardous compounds may be deposited to air, water and soil and negatively affect organisms and habitats. To protect the environment from pollution, laws have been implemented for reducing and maintaining levels of pollutants in the environment under safe thresholds. To follow up these legal implementations necessitates the development and usage of reliable and most

sensitive methods for detection of the various harmful and toxic compounds potentially released and found in the different media.

Chemical detection methods such as gas chromatography (GC) and other conventional chromatography methods can identify a specific compound by comparison with suitable references. Usually, they will also precisely measure the quantity of a given compound. However, such methods cannot reveal the toxicity of compound towards living organisms. Moreover, physical conditions like temperature, pH, presence of complexing ligands and other ions determine the bioavailability of a chemical compound. Whilst absolute concentrations of compounds are detected by chemical methods, this is not necessarily true for the bioavailability of it. Pollutants that are not bioavailable constitute only a potential but not an acute risk for the environment and the health of the organisms present in it (Reid et al. 2000, Hansen et al. 2001, Semple et al. 2003).

Biocontrol of hazardous compounds makes use of living organisms and their products. These respond to an individual toxic substance or a group of substances with similar harmful effects on the biological system and also effects of compounds of unknown chemical nature. Most importantly, they detect only the bioavailable pollutants, but not always in a specific way. Furthermore, biological test systems can access toxic effects of a sum of different hazardous compounds being individually present at very low concentrations (Rila & Eisentraeger 2003). The sensitivity and versatility of bioassays can thus exceed that of chemical methods. Biological assays are often technically simple compared to expensive and sophisticated chemical methods relying on sensitive modern technical equipment. When using organisms already available in a biotope, no extra costs for material arise in a biological approach. In certain instances, however, bioassays are mixed with chemical analysis, for example when determining quantities of heavy metals accumulated in living organisms (see examples in Table 1 and the text below).

Throughout the forest-wood-chain, there are specific needs for the evaluation of toxic compounds in environments via biocontrol. For example within forests, soil, air and water quality might be evaluated by biological measures (Table 1). Wood in processing as well as in service - especially when undergone chemical treatments related to its application - can be a source of various risks to the environment and health. Thus, pollutants from wood need to be monitored. Natural volatiles (see Chapter 11 of this book) emitted in larger quantities during wood processing might cause allergies - e.g., dermatitis was observed in increased frequencies in sawmill workers and employees within carpentry workshops (Eriksson et al. 2004). Treatments of wood with toxic compounds to repress microbial and insect attacks can also launch various environmental and health risks (Westlund & Nohrstedt 2000, Dahlgren et al. 2003, Dube et al. 2004, Stook et al. 2005, Chapter 13 of this book), for example through soil and groundwater contamination by leaching of toxic preservatives from wood in outdoor use or, after service, upon disposal at landfill sites (Khan et al. 2004, Townsend et al. 2005). In wood compo-

site production, glues of petrochemical basis can be a cause of health problems (Yang et al. 2001, Aalto-Korte et al. 2003, Fransman et al. 2003, Venn et al. 2003) in addition to various paints and varnishes used for protection and decoration of wood products (Salthammer 1997, Wieslander et al. 1997, Kaukiainen et al. 2005). Volatile organic compounds (VOCs) emitted from fresh wood and wood products have been suspected to contribute to asthma and the sick-building syndrome (Wieslander et al. 1997, Venn et al. 2003, Saijo et al. 2004, Kim et al. 2005), particularly in well-insulated renovated and newly built houses (Dingle & Franklin 2002, Diez et al. 2003, Emenius et al. 2004, Sakai et al. 2004, Sherman & Hodgson 2004, Gilbert et al. 2005, Kim & Kim 2005). Individuals can strongly vary in sensitivity (Runeson et al. 2004, Runeson & Norback 2005), so that in certain cases, ill-health symptoms occur under environmental conditions and under VOC concentrations that had been regarded as safe (Nakazawa et al. 2005). In many instances, the impact of compounds on health problems is still poorly documented (Burge 2004). Particularly in situations of very low concentrations of individual and mixed compounds, sensitive biological approaches might be better suited for detection and control of harmful substances in the environment than chemical methods.

Methods of biological monitoring distinguish **bioindicators**, **bioreporters** and **biosensors**. Bioindicators present naturally available organisms reacting in defined ways on changes occurring in their environment and probably have been appointed by experienced humans already in ancient times in evaluation of their environments. Careful observations and good environmental, organismal and ecological knowledge define such naturally available systems. Sophisticated modern bioreporters and biosensors employ biological material and electronical devices engineered by molecular biology, electrochemistry and physics (see also Chapter 11 of this book). The powerful potential of bioreporters and biosensors is likely only at the beginning of its exploitation for the detection of pollutants.

Bioindicators

All types of organisms, from prokaryotes and simple eukaryotes to highly developed plants and animals including humans, might be used as bioindicators (see Table 1 for examples). A bioindicator might be a species or a group of species that displays a narrow amplitude with respect to one or more environmental factors and responds predictably on environmental changes in a readily observable and quantitative way (Allaby 1992, McGeoch 1998).

To measure environmental pollution, an ideal bioindicator reacts most specific and very sensitive to (a) pollutant(s) and highlights the presence of (a) pollutant(s) by very obvious and unambiguous signals. Passive bioindicators are organisms already naturally present in a biotop (**detectors**), active bioindicators have been brought into an environment (**sentinels**) in order to study its pollution. Canaries

Tab. 1 Examples of bioindicators to detect pollutants in forest ecosystems

Bioindicator	Medium	Pollutant(s)	Bioindicator type	Effect	Reference(s)
Lichens	Air	Heavy metal fall outs, radionuclides, sulphur, nitrogen and phosphorus compounds, fluorides, chlorides, ozone, etc.	Response and accumulation	Compositional changes in lichen communities, accumulation of compounds within the thall	Confi & Cecchetti 2001, Luppi et al. 2003, Burka et al. 2004
Mushrooms	Soil	Ar, Cd, Co, Cu, Pb, Ag, Hg, etc., radionuclides	Accumulation	Accumulation of metals in the fruiting bodies	Yoshida & Muramatsu 1998, Blarusa et al. 2001, Falardey et al. 2002, Yoshida et al. 2004
		Nitrogen compounds	Response	Changes in macrofungal communities, transfer of nitrogen to phyto-bionts in ectomycorrhizal interaction	Trudell & Edmonds 2004, Trudell et al. 2004
Mosses and liverworts (<i>Bryum radiculosum</i> , <i>Fontinalis antipyretica</i> , <i>Conocephalum conicum</i> , <i>Pellia epiphylla</i>)	Air Soil Water	Cd, Cr, Cu, Pb, etc. fall outs Cr, Co, Cu, Ni, Ba, etc. Al, Fe, Cd, Cu, Zn, N etc.	Accumulation	Accumulation within the thall	Oymen et al. 1997 Schmitt et al. 2005 Vuori et al. 2003
Deciduous trees	Air	Level of ozone exposure	Response	Foliage injuries, morphological changes, accumulation of autofluorescent compounds	Chappellka et al. 1999, Ouellet et al. 2003, Novak et al. 2003, Manning & Godzik 2004, Buscotti et al. 2005
California black oak (<i>Quercus kelloggii</i>)	Soil	Nitrogen	Response	Foliar chlorosis	Gruke et al. 2005
Poplars (<i>Populus alba</i> , <i>Populus nigra</i>)	Soil	Cd, Zn, Pb, Fe, Zn, Cu, etc.	Accumulation	Accumulation within leaves	Medjón et al. 2004, Baslar et al. 2005
Pines (<i>Pinus halepensis</i> , <i>Pinus nigra</i> , <i>Pinus pinaster</i>)	Air	Nitrogen oxides, sulphur dioxide, ozone PAHs	Response Accumulation	Foliar discoloration, cellolysis, reduced phenol and flavonol concentration in needles, chlorotic mottle PAH accumulation	Pobles et al. 2003, Delslein & Vas 2005, Pizzardi et al. 2005

Tab. 1 (continued)

Bioindicator	Medium	Pollutant(s)	Bioindicator type	Effect	Reference(s)
Deciduous and coniferous tree barks and barks in combination with epiphytic lichen	Air, soil	Heavy metals, Mn, sulphur, acid pollutants, ozone	Accumulation and response	Deposition of pollutants in the bark, increase in the acidity of the bark, changes in bark lichen diversity, occurrence of bark beetles	Santamaria & Merin 1997, Bellis et al. 2003, Hauk 2003, 2005, Grodzki et al. 2004
Earthworms	Soil	Cd, Zn and other heavy metals	Accumulation and response	Accumulating in the body, avoidance of sites	Wiegmann 1991, Lukkat & Haimi 2005
Rabbit cistiode (<i>Mesogovius cistiodes</i>)	Air, soil	Cd, Pb, As, Hg	Accumulation	Accumulation in the parasite	Eira et al. 2005
Springtails (<i>Folsomia candida</i> , <i>Heteromurus nitidus</i>)	Soil	Acids, nitrogen, heavy metals	Response	Change in population density, soil decomposition activity	Kopaszki 1992
Honey bees	Air	Cd, Cu, Pb, Zn, etc. fallout	Accumulation	Accumulation within and on bees, in honey and royal jelly	Leita et al. 1996, Tuzen 2002
Damselflies (<i>Xanthocnemis zealandica</i>)	Water	Pesticides, insecticides	Response	Changes in levels of fluctuating asymmetry in wings	Henderson 2000
Fish (<i>Esox lucius</i> , <i>Perca fluviatilis</i> , <i>Rutilus rutilus</i>)	Water	Hg	Accumulation	Deposition in muscle tissue	Undestrom 2001, Sonesten 2001
Bird eggs (blue tit, <i>Parus caeruleus</i>)	Soil, water	Heavy metals	Response	Reduction of spermatozoa on the egg membranes	Dauwe et al. 2004
Bark vole	Air, soil, water	Heavy metals	Accumulation	Deposition in teeth	Gdula-Avguštica et al. 2004
Wild boars, deers, rabbits	Soil, water	Hg, Pb, chlorinated PCBs, Pb, fluoride	Accumulation	Accumulation in liver and kidney of the mammals, intestinal mucosa and muscles of rabbits, in antlers of deers	Hecht 1994, Guller et al. 1997, Dobrowolska & Meloniak 2002, Polomsky & Ribarik Luenik 2002, Eira et al. 2005, Nierdorf & Nierdorf 2006

for example served till 1986 in coal mines in Britain as sentinels for carbon monoxide that already at lowest concentrations in the air caused the birds to stop singing and to topple down. Two principle types of bioindicators to monitor type and levels of environmental pollution are distinguished. Response bioindicators (**responders** – this term is coined here in analogy to the nomenclature of other types of bioindicators in the literature) react with changes in their vital functions, accumulation bioindicators (called in the literature also **accumulators**) with depositing pollutants in their bodies (see Table 1 for examples). Response bioindicators define threshold levels of bioavailable pollutants in nature, upon which they give detectable reactions. Some response bioindicators show distinct positive reactions on the presence of a pollutant in the environment (known as **exploiters**). Algal bloom in over-fertilised, eutrophic water is an example for such a situation. More common, however, are negative effects by environmental pollutants on organisms such as physiological, morphological and behavioural stress symptoms (e.g. photosynthetic damage, tissue necrosis and enhanced susceptibility to pathogens in plants; see Table 1 for examples) and, in the worst cases, death of whole organisms and loss of species from a given biotope (names proposed here for such types of bioindicators: **sufferers** and **victims**). In most instances, reactions of response bioindicators can be registered by the naked eye and are thus easy to follow. In contrast, accumulation bioindicators collecting pollutants within the organism exhibit certain resistance towards the pollution stress. Such organisms are chemically analysed either in total or parts to quantify the accumulated toxic compounds. In case of fixed exposure times and a well-established indicative calibration, the level of environmental pollution can be quantified. Furthermore, accumulation bioindicators might give information on presence of pollutants below their critical thresholds response bioindicators react on. Reactions of bioindicators may be considered directly in their natural environment. However, it is also possible to develop specific bioassays in which the bioindicators are taken into the laboratory and exposed in defined ways to possibly contaminated matter (McGeoch 1998, Falla et al. 2000). Well established bioassays for testing fresh water quality are the *Daphnia* spp. and trout survival tests and the *Photobacterium phosphoreum* and *Vibrio fischeri* (Microtox®) bioluminescence tests (da Silva Nunes-Halldorson & Duran 2003; see below). Accredited bioassays for testing soil quality include earthworm mortality, seed germination and root elongation. For successful implementation of such bioassays, reactions of the organisms towards toxic compounds and the type of toxic compounds causing the reactions have to be clearly defined and test conditions to be standardised. Following sufficient assessment of the standardised tests proving their practicability and reliability, their usage might be approved on national and international level (Keddy et al. 1995, Cook et al. 2002, Braunbeck et al. 2005, Plaza et al. 2005). It is important to note that no universal bioassay is available for detection of all types of pollution, neither, there is a biological test that works under all circumstances for a given pollutant. For

instance, the very precise *Daphnia* water test reacts with a broad range of pollutants (metals, organic compounds), but copper toxicity is influenced by water hardness and dissolved organic carbon, requesting additional chemical tests for a most objective environmental risk assessment (Kramer et al. 2004, Long et al. 2004, Dewhurst et al. 2005).

Bioindicators in forestry

In forestry, bioindicators might be used as a measure to detect pollutants in soil, water and atmosphere (Collier et al. 2003, Madejón et al. 2004, Table 1), including pH changes (Gégout & Krizova 2003). Bioindicators might also serve as a measure of prevailing biodiversity and forecast changes in forest composition and habitat alteration (Rainio & Niemelä 2003, Duque et al. 2005). Some approaches regard the entire forest ecosystem as a receptor for pollutants [Critical loads and levels concept of the UN-ECE (United Nations Economic Commission for Europe), (UBA 2004)] or the implications for the sustainable management of forests (MCPFE 2002).

Mushrooms, functioning as accumulators, are particularly useful individuals for detection of various kinds of toxic metals and radionucleotides in the soil and outfall from the air, whilst overall mushroom communities can give insights into nitrogen abundance in forest soils (Yoshida & Muramatsu 1998, Trudell & Edmonds 2004; Table 1). Liver and kidneys of wild animals (e.g. wild boars, deers) are considered for evaluation of pollution by heavy metals but seasonal differences in nutrition (uptake of fungi in late summer and autumn) appear to affect the intake of the metals, limiting their value as bioindicators (Dobrowolska & Melosik 2002, Pokorný & Ribarič-Lasnik 2002, Pokorný et al. 2004). Likewise, accumulation of metals in the year rings of wood of trees is of limited reliability to follow up pollution since growth differences in annual increase, radial move of elements through ray parenchyma cells and mixed uptake of elements from soil and the air variably influence the outcome of subsequent chemical analysis of wood samples (Nabais et al. 1999, Bindler et al. 2004).

With rapid industrialisation and urbanisation, reactions of lichens to industrial air pollution have already been reported in the middle of the 19th century (Nyländer 1866). Since then, lichen communities serve in judging air qualities over wide areas, particularly in quantification of the SO₂ concentrations in the air. An established sentinel for ozone with carefully recorded reactions is presented by tobacco that is sensitive to higher levels of concentrations. Since 1980, also various forest trees in mountainous areas and national forests are used to judge pollution by SO₂, ozone and others (Falla et al. 2000, Verge et al. 2002, Table 1). Next to some perennial herbaceous plants (*Alchemilla* sp., *Astrantia major*, *Centaurea nigra*, *Centaurea scabiosa*, *Impatiens parviflora*, *Lapsana communis*, *Rumex acetosa*, *Senecio subalpinus*), shrubs (*Corylus avellana*, *Cornus sanguinea*, *Sambucus racemosa*) and vines

(*Humulus lupulus*, *Parthenocissus quinquefolia*), the tree species *Alnus incana*, *Pinus cembra* and *Sorbus aucuparia* are recommended ozone bioindicators in Europe (Manning & Gozik 2004). Regional scale ozone-impacts in the USA have been followed by foliar injuries (acute foliar injury, premature leaf loss) of trees at high risk such as *Liquidambar styraciflua*, *Pinus taeda* and *Prunus serotina* (Coulston et al. 2003). At a nationwide level, over thirty states of the USA participated from 1994 to 2000 in an ozone biomonitoring program (Smith et al. 2003) and ozone effects have been evaluated in a broader context with wet deposition of sulfate, nitrate, and ammonium to assess the global risks of air pollutants to forest health and vitality (Coulston et al. 2004).

The alarming forest decline ("Waldsterben") observed by crown thinning in Central Europe in the mid-1970 to early 1980s prompted integrated and ecosystem-oriented research programs on air and soil pollution in the German forests, in which the Faculty of Forest Science and Forest Ecology at the University of Göttingen had a leading position. Leaf loss and discolouring were described as effects of gaseous air pollutants and soil acidification as cause root damage resulting in water and nutrient stress (Ulrich 1990). Subsequently to these pioneering studies, visual assessment of defoliation and discolouration has been accepted in Europe as a standard method to evaluate forest health (UN-ECE 1998). In Italian forests for example, 4 main tree species (*Fagus sylvatica*, *Quercus pubescens*, *Quercus cerris* and *Picea abies*) were taken between 1997 and 2000 as indicators from sampling plots where the defoliation and discolouration of the crowns have been studied in response to pollution stress factors (Bussotti et al. 2002). In Austria since 1985 as a part of governmental programmes, the air pollution impact of SO₂ was studied countrywide in yearly analysis with the help of the Austrian Bioindicator Grid on 760 sample plots using as bioindicators *P. abies* (90 % of all assessed trees) and *Pinus sylvestris* (10% of all assessed trees). Sulphur concentrations of spruce and pine needles of the current and previous years are compared and the data used to control the emission of pollutants below the legal standards and enact measures for keeping air clean (Fürst et al. 2003, Smidt & Herman 2004).

Across Europe, the Level I monitoring program was launched in 1986 to monitor annually in forests the crown condition on a systematic transnational grid of 16 x 16 km, complemented by assessments on national grids of varying densities. Between 1992 and 1996, the soil condition and the foliar nutrient status were also recorded. Since 1994, in selected European forest ecosystems soil and soil solution chemistry, foliar nutrient status, increment, meteorological condition, ground vegetation and deposition of air pollutants are measured (Level II monitoring) in addition to the annual crown condition assessments. The intensive level II sectorial monitoring interconnects with the level I monitoring on an area representative scale (see ICP Forests, International Co-operative Programme on assessment and Monitoring of Air Pollution Effects on Forests; <http://www.icp-forests.org/Objectives.htm>). In Germany, there are 86 Level II plots (stand 1st of

January 2000) that are annually evaluated according to the regulation of the European Programme for the Intensive Monitoring of Forest Ecosystems (EC 1998; AG DBF-WS 2001). Defoliation is estimated in 5% classes for individual trees of main species, *P. abies*, *Pinus sylvestris*, *F. sylvatica* and *Quercus robur et petraea*. Crown condition as surveyed by the level of defoliation is a core parameter in forest condition reports (BMVEL 2004). Stand-age, biotic factors (phytophagous insects, fungi), geographical place, time of survey within the year, etc. will influence the relative outcome from monitoring defoliation. Considering additional parameters from evaluation of Level II plots allows integrated analysis of complex relationships in different ecological situations and their temporal variation. Stress effects on the complex forest systems by direct or indirect impact of air pollutants and soil acidification are expected to become more transparent (Seidling 2004, 2005, Seidling & Mues 2005).

The concept of identifying critical threshold of anthropogenic pollutant depositions for forest ecosystems was introduced in 1979, by the UN-ECE "Convention on Long-Range Transboundary Air Pollution" (UN-ECE 1979). Nilsson & Grennfeld (1988) defined the critical load as "a quantitative estimate of an exposure to one or more pollutants below which significant harmful effects on specified sensitive elements of the environment do not occur, according to the present knowledge". The exceedance of critical loads is calculated as the difference between the deposition and the critical load. It is assumed that long-term exceedance of critical loads will have effects on the functioning of the entire forest ecosystem (UBA 2004). Critical loads (CL) are calculated for long-term steady state conditions. The maximum tolerable input for acidifying substances (CLmaxS) is determined using the criterion "prevention of the depletion of secondary Al phases". This criterion is expressed as critical limits, i.e. the critical concentration ratio of aluminium and base cations in the soil solution. The tolerable input of eutrophying nitrogen (CLnutN) is related to the nitrogen retention in the forest ecosystems avoiding considerable nitrate leaching from forest soils (UBA 2004).

Calculations of critical loads for acidifying inputs and nitrogen and their exceedance were compared to different indicators of the soil and forest conditions in the German part of the extensive forest monitoring (Level I and II plots; Augustin et al. 2005). The results support the concept of critical thresholds in that way that their exceedance can impair forest ecosystem functions like nitrogen retention in forest ecosystems: for Norway spruce sites, high exceedance of critical loads for nitrogen and nitrogen deposition corresponded well with low C/N humus. However, the crown condition was only weakly positively related to indicators for acidifying sulphur inputs like the sulphur content in tree leaves and needles. This points to the difficulties to relate crown condition to acidifying deposition as single effect factor and ignore interaction terms between meteorological stress variables, soil variables, and bio-geographic regions (De Vries et al. 2003).

Another forest ecosystem approach for bioindication was established by the Ministerial Conference on the Protection of Forests in Europe (MCPFE) when introducing quantitative and qualitative pan-European indicators for sustainable forest management (MCPFE 2003). Quantitative indicators were attributed to six criterions evaluating maintenance and, where applicable, enhancement of forest resources and their contribution to global carbon cycle, forest ecosystem health and vitality, productive functions and biological diversity of forests, enhancement of protective functions in forest management and other socio-economic functions and conditions. The indicators cover a wide range of forest ecological, nature conservation, silvicultural and socio-economical parameter that are completed by a qualitative assessment of political actions. Ongoing forest certification of sustainable forest management in Germany and Europe mainly base on the MCPFE indicators (PEFC 2005).

Bioindicators in applications of wood industry

Whilst in the forests potential bioindicators for evaluation of environmental pollutants are naturally large in numbers, there are only few reports dealing with bioindicators for pollution problems caused specifically by the wood industry and the usage of wood. Outdoors, the same sets of organisms might be appointed as for other pollution problems (see above).

As a specific problem, the wood preservative creosote, a mixture of many chemicals including polycyclic aromatic hydrocarbons (PAHs) used to protect marine pilings, railway sleepers and utility poles (see Chapter 13 of this book), leaches into aquatic environments with the consequence of zooplankton reduction and increase in algal growth (Sibley et al. 2004). Destruction of photosynthesis (chlorophyll-a fluorescence) of the aquatic plants *Myriophyllum spicatum* and *Lemna gibba* has been tested as another bioindicator for creosote in water (Marwood et al. 2001, 2003). GC-MS analysis of extracts from pine needles taken from wood-preserving sites were used to monitor the degree of PAH contamination at such places (Safe et al. 1992). Measurement of bile material from fishes for PAH content has also been suggested for detection of these type of pollutants in aquatic environments (Ariese et al. 2005). A further method is the molecular detection by 16S rRNA amplification (see Chapter 9 of this book for technical details) of a PAH-degrading *Sphingomonas* species that specifically occurs in contaminated soils (Leys et al. 2005).

Effluents from the pulp and paper industry contain endocrine-disrupting substances with effects on reproduction rates and secondary sex characteristics in fish (e.g. fathead minnow, *Pimephales promelas*) that can be detected in a concentration-dependent manner by the very sensitive reactions of the fishes (Parrott & Wood 2002, Parrott et al. 2003, 2004, Kovacs et al. 2005). Genotoxic effects of wood dusts on DNA have recently been recorded in human lymphocytes in a test

system referred to as Comet assay (Faust et al. 2004). The same method can be applied in detecting effects from burning wood (Pandey et al. 2005) but age, gender and smoking habits of test persons have to be considered when interpreting the results (Møller et al. 2000). A urinary assay is an alternative to measure phenolic compounds taken in with wood smoke (Dills et al. 2001). Within houses, asthma and the symptoms of the sick building syndrome might be biological indications for hazardous volatile emission from wood and wood products (Jensen et al. 2001). However, there are many other sources in indoor environments causing alike symptoms, such as cigarette smoke, fungal volatiles produced by moulds and inks in offices handling much paper work (Venn et al. 2003, Burge 2004). Proper ventilation can help to overcome acute symptoms (Burge 2004) but long-term exposition below concentrations causing acute symptoms might still affect human health, requesting for suitable sensitive assessments.

Bioreporters and biosensors

Bioreporters are whole cell organisms that respond to environmental conditions by up- or downregulation of certain genes that code for easily recognisable and measurable proteins under control of promoters (regulatory DNA sequence localised in front of a gene) responding specifically to a signal (Fig. 1). Such genes are referred to as **reporter genes** and their products as **reporter proteins** whose

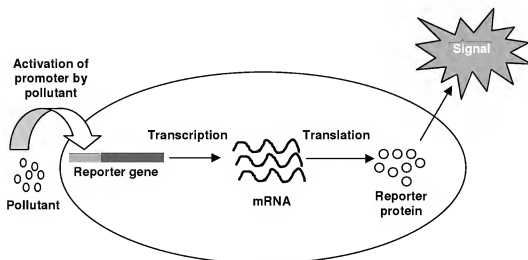


Fig. 1 A bioreporter responding via gene transcription and mRNA translation with the production of detectable protein upon sensing a pollutant (modified from Lewis et al. 1998). The promoter of the reporter gene is indicated in grey, the coding region for the protein product in black. The protein might either be directly detectable in concentration by specific properties (for example the green fluorescent protein) or indirectly by enzymatic activity (for example luciferase)

quantity is influenced by the strength of a signal. Such a signal can be the environmental concentration of a pollutant or a group of pollutants. Natural or genetically engineered whole cell organisms are used as bioreporters for detection of toxic compounds. Most commonly, the bioreporter is a microbe, either a bacterium or a yeast. Usage of microbial bioreporters has the advantages that reactions are rapidly, easily and precisely recorded. Thus, microbial bioassays are increasing in popularity for toxicity measurement of environmental pollutants on living organisms.

Natural bioreporters

Common natural bioreporters are the marine bioluminescent bacteria *V. fischeri*, *Vibrio harveyi* and *P. phosphoreum* that possess *lux* genes for an enzyme called luciferase. This enzyme catalyses a biochemical reaction in which the energy-rich reduced flavin-mononucleotide FMN_{H2} reacts with oxygen and a long-chain aldehyde and is converted into FMN, water and a fatty acid. By this enzymatic reaction, energy is released in the form of a blue-green light at 490 nm. In *V. fischeri* and *V. harveyi*, *lux* genes are autoinducible by the self-produced diffusible pheromones β -ketocaproyl N-homoserine lactone and β -hydroxybutyryl-N-homoserine lactone, respectively. This phenomenon of autoinduction of bioluminescence is known as Quorum sensing. The expression of *lux* genes and thus the efficiency of light emission depend upon the cell densities. Bioluminescence increases with cell densities. Depending on their level of toxicity, pollutants in bacterial cultures inhibit the metabolic activities and thus energy production of the cells or even kill the cells, resulting in both cases in a decrease of bioluminescence (Meighen 1991, 1993, da Silva Nunes-Halldorson et al. 2003; Fig. 2). This principle is used in various commercial bioluminescence assays (Microtox, ToxAlert, LUMISTox). These worldwide accepted systems detect a broad range of pollutants by decrease of bioluminescence in a defined bacterial suspension of high cell densities in a saline nutrient-rich solution by measuring emitted photons in a luminometer (Jennings et al. 2001, da Silva Nunes-Halldorson et al. 2003; Fig. 2). Contamination by toxic wood extractives of process and waste waters from pulp and paper industries and of lake sediments in areas with paper mills have successfully been monitored by measuring bioluminescence inhibition of the bacterial test organism (Kostamo & Kukkonen 2003, Rigol et al. 2004, Lahdelma & Oikari 2005), and PAHs and phenols from combusting wood and coal tar (Pimenta et al. 2000, Loibner et al. 2004).

In ten-thousand-fold diluted cultures of *V. fischeri*, *V. harveyi* and *Photobacterium* species, significant induction of bioluminescence is observed upon treatment with mutagens like sodium azide, 2-methoxy-6-chloro-9-acridine, 4-nitro-o-phenylenediamine, 4-nitroquinolone-N-oxide, 2-amino-fluorene and benzo(a)pyrene, the latter of which is a known wood preservative. Like UV-irradiation, upon damage of the bacterial DNA these mutagens induce *lux* gene expression by the SOS

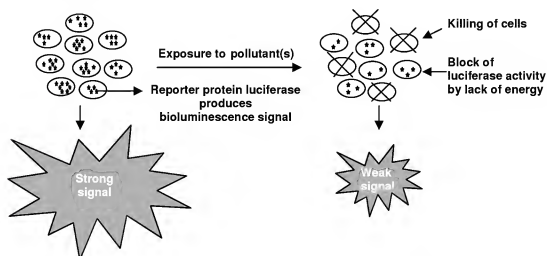


Fig. 2 The bacterial bioluminescence system: the toxicity of pollutants can be determined by a decreased intensity of a bioluminescence signal upon block of cell respiration of the bioreporters *Vibrio fischeri*, *Vibrio harveyi* or *Photobacterium phosphoreum*

response through inactivation of the LexA repressor bound to various promoters including that of the *lux* genes. Therefore, measuring increase of bioluminescence in diluted cultures has been proposed as a test system for the assessment of mutagens at concentrations below toxicity levels (Czyz et al. 2002).

Bioluminescence is not only observed in bacteria but for example also in various basidiomycetes such as *Armillaria mellea* and *Mycena citricolor*. Mycelia of these two fungi have been shown to reduce their levels of bioluminescence in response to exposure to the PAH 3,5-dichlorophenol (3,5-DCP), the wood preservative pentachlorophenol (PCP) and Cu, respectively. The sensitivity of the reaction equals that of bacterial bioluminescence tests, showing that these organisms also can act as natural bioreporters (Weitz et al. 2002). Such fungal systems could be of particular interest when wanting to test toxic effects of compounds to wood-rotting fungi, most of which are basidiomycetes.

Genetically engineered bioreporters

The level of sensitivity for a given bioreporter against a pollutant generally depends on its uptake into cells and this can differ between organisms. Changing the organism in which gene expression takes place can therefore increase the range and/or the efficiency of scoring detectable pollutants. The natural *V. harveyi lux* genes have been transferred into *Streptomyces lividans* and shown to be active in this bacterium with much higher sensitivities for some toxic metals and phenols (Park et al. 2002). Changing promoters by genetic engineering can alter the range of specificity of the *lux* system to react only on pollutants of specific interest (Fig. 1). In this way, a luciferase assay in *Escherichia coli* restricted to arsenic salts has been

developed for detection of the wood preservative chromated copper arsenate (CCA) at sites where CCA-treated wood has been used. The detection limit of the system was determined at 0.01 $\mu\text{g As/ml}$ (10 ppb) well within the range of environmental concern. However, if needed, the sensitivity of the assay can be further increased by phosphate limitation (Caj & DuBow 1997). Various other bacterial promoter-*lux* gene constructs are available in *E. coli* and other bacteria to measure specifically other environmental pollutants or groups of contaminants in the environment (Köhler et al. 2000, Gu et al. 2004), several of which are also relevant to the wood industry (heavy metals, various organic pollutants).

Indeed, most of the bioreporters nowadays in use are produced by genetic engineering in which an environmentally and metabolically responsive promoter is fused to a suitable reporter gene and introduced into an appropriate microbial host either by plasmid or chromosomal insertion. Changes in the amount of reporter protein are an indication of changes in the transcriptional activity of the promoter, which in turn depends on the stimuli to which the promoter is responding (Leveau & Lindow 2002). Reporter genes other than the bacterial *lux* genes are the *luc* gene coding for insect luciferase (Lagido et al. 2001), *gfp* from the jelly fish *Aequorea victoria* encoding green fluorescent protein (March et al. 2003), the *E. coli* *lacZ* gene which codes for β -galactosidase that converts the colourless artificial substrate X-gal (5-bromo-4-chloro-indolyl- β -D-galactoside) into blue coloured 5-bromo-4-chloro-indigo (Guarente 1983), an *E. coli* gene encoding β -glucuronidase is an exohydrolase that catalyses the hydrolysis of β -D-glucuronides into D-glucuronic acid and aglycone (Gilissen et al. 1998, Sarker & Nakar 2003, Basu et al. 2004) and *pboA* for an alkaline phosphatase that converts 4-methylumbelliferyl phosphate (MUP) into fluorescent and stable product 4-methylumbelliferone (4MU) (Femley & Walker 1965, Köhler et al. 2000). In most instances, fast growing prokaryotic organisms serve as expression hosts for these various reporter genes, also because there are many kinds of promoters available reacting to specific matters. However, prokaryotic cells have limitations in their application to evaluate the toxicity of chemicals towards the eukaryotic organisms. Therefore, other whole cell bioreporters are developed based on eukaryotic organisms like yeast (Hollis et al. 2000, Gupta et al. 2003). For example, the bacterial *lux* genes have been combined with an estrogen-sensitive promoter in the yeast *S. cerevisiae* and this system can now be used in sensing of environmental contamination by estrogens (Sanseverino et al. 2005). This system can be of relevance e.g. for controlling flue gas streams from incomplete combustion of wood and effluents from pulp and paper mills, known to likely contain harmful estrogenic compounds (Mellanen et al. 1999, Muthumbi et al. 2002). Currently, a specific yeast-based human estrogen receptor (hER) bioassay is used for such a purpose (Muthumbi et al. 2002, 2003, Svenson & Allard 2004, Wang et al. 2005). In hER-based yeast reporter systems, the human estrogen receptor is expressed from a gene inserted into the yeast's genome whilst an estrogen-responsive promoter controls

expression of the bacterial *lacZ* reporter gene from a plasmid. Upon binding of estrogen to the estrogen receptor, the complex interacts with the estrogen-responsive promoter elements to initiate reporter gene transcription (Routledge & Sumpster 1996, De Boever et al. 2001). The CALUX system uses recombinant mouse and rat cell lines in which expression of the firefly luciferase gene is controlled by dioxin-responsive elements and is currently the best system for detection of dioxin and dioxin-like compounds (Hoogenboom et al. 2006). The system has been applied to determine dioxin contamination in risk assessment of recycled wood for the use as animal bedding (Asari et al. 2004).

Generally, the choice of a promoter depends on the kind of substance to be determined by a bioreporter. Genomes contain thousands of genes. All the organisms from simple eukaryotes to highly evolved eukaryotes have developed some metabolic pathways against different stress conditions. These pathways are stress-specific. Several different genes can be up- or down-regulated under a specific stress condition. For example, in the yeast 854 genes respond to heat shock at 37°C representing about 15% of the yeast genome. Specific genes are induced in all organisms by natural and anthropogenic stressors (Causton et al. 2001). Gene expression profiles in stress response against a specific toxin can be established with various highly sophisticated molecular techniques such as microarray analysis, differential display polymerase chain reaction (DD PCR), suppressive subtractive hybridisation PCR (SSH PCR), real competitive PCR, and serial analysis of gene expression (SAGE) (Ahmed 2002, Fryer et al. 2002, Liang et al. 2002, Snell et al. 2003, Ding & Cantor 2004, Pemmasani 2006). It is therefore feasible to characterise patterns of stress induced gene expression and correlate them to a particular stressor to identify upon need either a suitable promoter reacting on a specific compound or on a larger group of pollutants (Pemmasani 2006). Furthermore, rapidly growing sequence databases provide the opportunity to select more suitable and accurate promoters even from non-model organisms also (Snell et al. 2003). Genomic revolution certainly opened the way for the construction of more efficient and larger number of bioreporters.

Biosensors

The terms bioindicator, bioreporter and biosensor are not uniformly used within literature and, therefore, three principally different types of biological monitoring are often confused with each other. According to the definition by the IUPAC (International Union of Pure and Applied Chemistry), biosensors are the highly sophisticated, most sensitive electrochemical devices that bring bioindicators or natural or engineered bioreporters (the biorecognition element, receptor) in spatial contact with an electrical device (transducing element usually linked to an amplifier and a computer unit) to measure toxic substances most specifically and accurately even at lowest concentrations and provide quantitative or semiquantitative

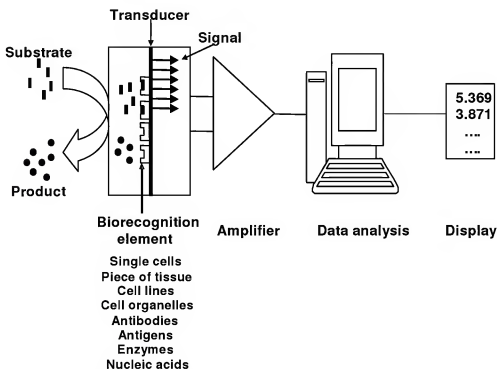


Fig. 3 The principle of biosensors. Chemical compounds are led to a biorecognition element (the most common are listed in the figure) where they undergo specific reactions (e.g. an enzymatic conversion) giving a product and/or energy. Upon recognition by a specific transducing element (transducer), the product or the energy is transformed into a specific electric signal. This signal will be amplified by an electrical device and transferred to a computer, where it will be processed by suitable computer programs to finally display a measuring value corresponding to the quantity of the chemical compound in the original sample

analytical information (Fig. 3). More easily expressed, a biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals (IUPAC 2007; see also Chapter 11 of this book). Multi-use biosensors possess the ability to be repeatedly calibrated and can be used continuously in a reliable way, unlike bioreporters that are discharged or disposed after having been used. The whole cell bioreporters whose reactions are measured for example upon insertion of the cells into a photometer or a luminometer, should therefore clearly be distinguished from the electrochemical biosensors (Thevenot et al. 2001).

The production of biosensors involves the application of conventional engineering technology and, in many instances, gene technology as well. There are different kinds of biorecognition elements and of transducing elements. The bio-

recognition element can be whole cells (single microbial cells, plant tissues, cell lines from animals, etc.), antigens, antibodies, enzymes, DNA and others. A transducing element can be electrodes measuring the current resulting from electrochemical oxidation or reduction of an electroactive chemical species (amperometry), ion-sensitive electrodes measuring the potential difference between an indicator and a reference electrode or between two reference electrodes separated by a permselective membrane (potentiometry), ion-sensitive field-effect transistors, parallel ion conductometric devices, piezoelectric transducers, photocells of light absorption and reflectance and others (Zhai et al. 1997, Thevenot et al. 2001, Rodriguez-Mozaz et al. 2004). The selection of both the biorecognition element and the transducing element are critical in the construction of an efficient biosensor.

Microbial whole cells are proven to be amenable for immobilisation in a biosensor configuration. In the recent years, various whole cell biosensors have been developed as tools to detect and quantify the toxicity of samples from different environments. In most instances, they are recombinant organisms carrying reporter genes behind promoters inducible by specific signals, such as environmental pollutants (Bentley et al. 2001, Hansen & Sorensen 2001, Keane et al. 2002, Gu et al. 2004). Thus, microbes might be incorporated in biosensors that are elsewhere plainly employed as bioreporters.

Within the wood sector, biosensors employing specific enzymes are of interest. The chromate reductase from the sulphate-reducing bacterium *Desulfomicrobium norvegicum* was used to develop an amperometric biosensor to measure chromate bioavailability (Michel et al. 2003). Many efforts have gone into developing biosensors with fungal laccases as the biorecognition element. The enzymes are for example mounted in form of cross-linked enzyme crystals into electrodes yielding sensors that keep good activity for over three months (Roy et al. 2005). In other cases they are immobilised on the surface of spectroscopic graphite electrodes (Haghighi et al. 2005) or negatively and positively charged glassy-carbon electrodes (Yaroplov et al. 2005). Laccases from various species as for example *Trametes versicolor*, *Trametes hirsuta*, *Rigidoporus lignosus* are applied (Vianello et al. 2004, Haghighi et al. 2005, Roy et al. 2005) including one recombinantly produced enzyme (Kulys & Vidziunaite 2003), to detect all kinds of phenolic compounds in the environment including phenolic compounds from wood and paper industries (Freire et al. 2001). Determining best reaction conditions with various phenolic substrates for the different laccase enzymes and optimising storage conditions and the enzymatic active life-times of electrodes are busy fields of research (Freire et al. 2001, Yaroplov et al. 2005).

Conclusions

In this paper, we present insights into the enormous potential of using biological materials in detecting pollutants in forests, from wood industries and from wood

in usage. Often, the presented biological tests are still at a test stage. The best suitable systems have to be chosen considering criteria such as sensitivity and reliability, but also ease of handling and economical costs. Bioreporter and biosensor systems can be further developed or newly invented. Accreditations have to follow and test standards to be implemented. Avoidance of usage of toxic compounds wherever possible (see Chapter 13 of this book) however would reduce our need for such biomonitoring systems.

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Part IV – Wood Preservatives and Wood Protection

13. Wood Preservatives

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Introduction

Wood preservatives are chemical preparations that prevent the infestation of wood through degrading and staining fungi, insects or marine organisms (**preventive wood preservation**) or, in case of already occurred infestation, are used for remedial treatment. According to the definition of the Technical Committee 38 (TC 38) as part of the European Committee for Standardisation (CEN; <http://www.cenorm.be/cenorm/index.htm>), all wood preservations contain biocides as active ingredients.

The European Directive 98/8/EG (**Biocidal Product Directive**, 1998) regulates the approval and marketing of biocidal products. In Germany, preventive chemical wood preservation is regulated by the DIN 68800-3. The standard assigns hazard classes, closely related to the European standard EN 335-1 (1992),

Table 1 Hazard class system according to EN 335-1 (European standard EN 335-1 1992) and biological agents according to Hughes (2004)

Hazard class (HC)	Service conditions	Exposure to wetting	Biological agents
1	Without soil contact covered (dry)	Non	Insects
2	Without soil contact covered (risk of wetting)	Occasionally	Insects, decay fungi
3	Without soil contact uncovered	Frequently	Insects, decay fungi, disfiguring (bluestain) fungi
4	With soil contact	Permanent	As HC-3 plus soft rot fungi
5	Salt water contact	Permanent	As HC-3 plus marine borers

which will be changed in course of revision into use classes following prEN 335-1 (2004) (see Table 1).

In Germany, wood preservatives for load bearing components require an official approval by the Deutsches Institut für Bautechnik (DIBt, German Institute for Building Technology; <http://www.dibt.de/>). For this approval, the efficacy related to a particular hazard class is evaluated on the basis of submitted results from required standard tests. In addition, the Bundesinstitut für Risikobewertung, (BfR, Federal Institute of Risk Assessment; <http://www.bfr.bund.de/>) evaluates the health risks and the Umweltbundesamt (UBA, Federal Environmental Agency; <http://www.umweltbundesamt.de/>) evaluates the environmental compatibility of the respective preservative. Along with the approval, the following quality grades are assigned:

- Iv: preventively effective against insects
- P: effective against fungi (rot protection). This grade is only assigned when preventive effectiveness against insects has been demonstrated
- W: for wood exposed to weathering, however, which is not in permanent ground contact or permanent contact with water
- E: wood exposed under extreme conditions (ground contact, running water, etc.)
- (P): effective against fungi (for wood composites)
- Ib: remedially effective against insects
- M: Schwammsperrmittel (preservatives for barrier treatment)

Wood preservatives for **non-load bearing wood components**, such as windows, claddings or fences may be evaluated by a voluntary inspection procedure of the Gütegemeinschaft Holzschutzmittel e.V. (Quality Assurance Association for Wood Preservatives; <http://www.holz-schuetzen.de/>) which awards the “Gütezeichen RAL Holzschutzmittel” (quality mark for wood preservatives) based on test results and evaluation by the BfR and the UBA.

For the approval of a wood preservative in Germany, the Chemikalien-verbotsverordnung (1993, Chemicals Prohibition Order) has to be considered with regard to a ban [e.g. on pentachlorophenole(PCP)] or a restriction on the application of certain preservatives (such as creosotes).

Wood preservative formulations

Wood preservative formulations are classified in terms of their chemical constitution:

- Tar oils (creosotes)
- Water soluble/water-borne preservatives
- Organic solvent-borne preservatives
- Emulsions/micro-emulsions

Tar oils (creosotes)

Tar oils are complex chemical mixtures that predominantly consist of aromatic hydrocarbons. These derive from the distillation of coal tar at a boiling temperature range of 200–400°C (Richardson 1993).

The combination of many chemical ingredients provides a broad spectrum of efficacy and a long-term protection against fungi (particularly soft rot), insects and wood-decaying marine organisms. Its mode of action is mainly based on the presence of polycyclic aromatic hydrocarbons (PAH; Table 2) and various phenolic compounds. Some of these components exhibit synergism, so that the toxicity of individual components is probably not of major significance (Nicholas 2001). In addition to the biocidal action, tar oils render wood hydrophobic (water repellent) and reduce the moisture absorption of treated wood (Komora 1999).

Health hazards, brown staining, a strong smell and a poor paintability and gluing ability are the crucial drawbacks of tar oils. Many of the PAHs in tar oils are evaluated as harmful to health and as potentially cancerogenic (European Directive 2001/90/EG). Due to these properties, tar oils exclusively suit for outside applications, particularly for wood permanently exposed to ground or water contact, i.e. in hazard class 4 (railway sleepers, poles, shoreline stabilisation, etc.) and in hazard class 5 (contact to sea water) according to the European standard EN 335-1 (Lohmann 2003).

Table 2 Content of polycyclic aromatic hydrocarbons (PAH) in tar oils of WEI-type A and C (Komora 1999)

Component	WEI-type A	WEI-type C
Naphthalene	8.7	0.1
2-Methyl-naphthalene	6.5	0.4
1-Methyl-naphthalene	4.0	0.3
Acenaphthene	8.0	2.4
Dibenzofurane	5.7	0.7
Flourene	5.8	2.0
Phenanthrene	7.4	26.2
Anthracene	1.0	1.5
Flouranthene	5.0	11.2
Pyrene	4.8	5.2
Benz(a)anthracene	0.7	0.01
Chrysene	0.6	0.01
Benzo(e)pyrene	0.04	0.002
Benzo(a)pyrene	0.05	0.003

In the European Union, the application of tar oils is restricted to industrial impregnation processes (European Directive 2001/90/EG). The West European Institute for Wood Impregnation (WEI, <http://www.wei-ieo.org/>) is assigned with quality control and specification of tar oils in Europe. The maximal content of the PAH benzo(a)pyrene (BaP) in tar oils is the crucial factor for their usage and field of application. It depends on the progression of the distillation curve. Typical distillation curves are depicted in Fig. 1. Tar oils are classified into WEI-Types that differ in terms of their distillation curves and, thus, in their physical properties and BaP content (Komora 1999):

- **WEI-type A** is a heavy oil (high density) used for railway sleepers with a high content in the high boiling fraction and a BaP content of less than 500 mg kg⁻¹. The European directive 2001/90/EC prohibits all tar oils exceeding a BaP content of 50 mg kg⁻¹ since June 2003 and recommends a future substitution by WEI-type C.
- **WEI-type B** is a light oil from the low and medium boiling area with a BaP content lower than 50 mg kg⁻¹ which is mostly used for poles.
- **WEI-type C** is an oil from the medium boiling area with minor smell intensity and low BaP content (5 - 50 mg kg⁻¹) in which the low and high boiling distillate fraction were separated.

Carbolineum is a light mixture of various tar oil components which can be handled and processed at ambient temperature. Nowadays, carbolineum is a

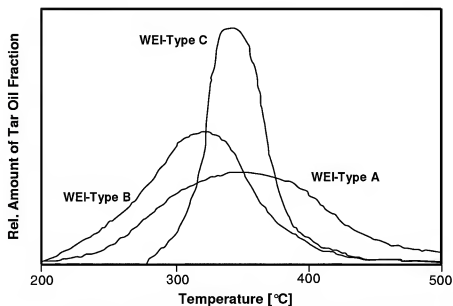


Fig. 1 Distillation curves of WEI tar oil types (Producer information Rütgers Organics, Mannheim)

collective term in the German-speaking part of Europe for tar oils which were specially produced for simple treatment techniques, in order to distinguish the product from tar oils exclusively applied for industrial processes (Willeitner and Dieter 1984). Its BaP content is currently below 50 mg kg^{-1} . Due to its low viscosity, it was formerly also mixed with organic solvents and used for painting; however, it is currently prohibited for non-professional application in the European Union (European Directive 2001/90/EG, Anonymous 2000).

GX plus is a relatively new product (1996) based on WEI-type C tar oil, blended with mineral oils in order to improve its treatability (Komora 1999).

Pigmented emulsified creosote (PEC), recently introduced in Germany, is an oil-in-water emulsion which contains 80% tar oil of WEI-type C and pigments. PEC displays the advantage that it is easier to handle than common tar oils, makes a smoother and less sticky surface and does not easily exude from wood (Lohmann 2003, Chin et al. 1983).

Water soluble/water-borne preservatives

Water soluble preservatives are commonly pure inorganic salts or salt mixtures in the form of pastes or as liquid concentrates. In addition, inorganic ingredients are currently often combined with organic agents. The latter can be diluted or are dispersed in water by adding specific compounds/carrier solutions to the formulation - that is why there are called "water-based" (Irmschler & Quitt 2005). Water soluble/based preservatives are particularly suited for wet wood or wood with

Table 3 Classification of water-borne wood preservatives in Germany and their applicability in different hazard classes (based on Irmschler & Quitt 2005)

Preservative type	Effective ingredient	Efficacy	Hazard classes
B-salts	Boron compounds	P, Iv	1, 2
SF-salts*	Silico-fluorides	P, Iv	1, 2
CFB-salt**	Chromium-fluorine-boron comp.	P, Iv, W	1, 2, 3
CC-salts	Copper-chromium comp.	Iv, W, E	1, 2, 3, 4
CCA-salts**	Copper-chromium-arsenic comp.	P, Iv, W, E	3, 4
CCB-salts	Copper-chromium-boron comp.	P, Iv, W, E	1, 2, 3, 4
CCF-salts**	Copper-chromium-fluorine comp.	P, Iv, W, E	1, 2, 3, 4
CCFZ-salts*	Copper-chromium-fluorine-zinc comp.	P, Iv, W, E	1, 2, 3, 4
Quat ¹ preparations	Quaternary ammonium comp.	P, Iv, W	1, 2, 3
Quat-boron preparations	Quaternary ammonium /boron comp.	P, Iv, W	1, 2, 3
Chromium-free copper preparations	Copper-HDO ² , quats ¹ , triazoles	P, Iv, W, (E)	1, 2, 3, (4)

¹ quat: quaternary ammonium compound; ² HDO: bis-(N-cyclohexyldiazoniumdioxo)

* No more in use; ** scarcely used

a moisture content below the fibre saturation point. The preservative systems presented in Table 3 are classified in terms of their fixation in wood.

The most important inorganic ingredients in wood preservative formulations are **copper compounds**. Apart from tar oils, copper salts are the only preservation agents that are able to protect wood in soil contact since they exert a biocidal action against soft rot fungi (Hulme & Butcher 1977). However, copper-chromium salts are little effective against copper-tolerant brown rot fungi (*Poria* family) which are able to detoxify copper salts by chelation with oxalic acid (Da Costa 1959, Da Costa & Kerruish 1964, Collett 1992, Woodward & De Groot 1999). In order to combat copper-tolerant brown rot fungi, further agents such as boron (boric acid, borax, boric acid ester), quaternary ammonium compounds (quats), organic biocides, and scarcely arsenic (arsenic pentoxide, arsenate, arsenic acid), or fluorine salts (fluoride) are added to the formulation; most of these are also active against insects (Irmschler & Quitt 2005).

The main task of chromium in preservative formulations is to cause the fixation of other active ingredients. In these formulations, chromium exists in the form of orange-red Cr(VI)-compounds which are toxic and cancerogenic. Fixation of other biocidal salts is caused *via* oxidation of the wood constituents and the formation of inorganic precipitates. Arsenic is fixed primarily by trivalent chromium, probably as chromium arsenate (CrAsO₄) or copper arsenate (e.g.

$\text{Cu}(\text{OH})\text{CuAsO}_4$). Excess copper, which does not form a complex with lignin or carbohydrates, reacts with dichromate to produce chromates such as CuCrO_4 (Richardson 1993, Nicholas 2001). Concomitantly with the oxidation of wood components, $\text{Cr}(\text{VI})$ is reduced to the less toxic and non-cancerogenic $\text{Cr}(\text{III})$ which is – together with copper salts – responsible for the typical grey-green colour of chromium treated wood (Lohmann 2003). Chromium (III) shows only a minor biocidal activity towards fungi and insects. Chromium also protects wood from degradation through UV radiation and weathering (Evans & Schmalzl 1989) and inhibits corrosion (Richardson 1993).

Due to their toxicological properties, the application of chromium-containing preservatives is currently restricted in many countries and a ban is discussed for the future. Occupational health and safety as well as elaborate and costly waste management of the chromium treated waste wood are the main reasons for the intended ban (Helsen & Van den Bulck 2005). According to the “Technical Rules for Hazardous Substances” TRGS 618 (Substitutes and Application Restrictions of Wood Preservatives Containing Chromium[VI], 1997), the application of chromium-containing products is restricted to immersion/steeping and vacuum-pressure impregnation and, thus, only for outdoor application.

Arsenic compounds are classified as very toxic and cancerogenic and are therefore not used anymore in Germany (Lohmann 2003, European Directive 2003/2/EG). Disposal of copper-chrome-arsenic (CCA)-treated waste wood is another problem which raised concerns because of leaching of preservatives on landfills and possible release of arsenic during combustion (see Mai et al. 2004).

In Germany, the major chromate-free fixating wood preservatives comprise copper-HDO [Cu-HDO ; bis-(N-cyclohexyldiazoniumdioxo)-copper], various copper-quat preparations (CuQuat , CuQA) and copper-triazoles (CuAz). Cu-HDO shows activity against soft rot as well as white and brown rot fungi (basidiomycetes). Activity against basidiomycetes is generally based on the complexing agent HDO. Aluminium-HDO (only in organic solvents) and potassium-HDO (only for wood composites) also protect against white and brown rot decay, but not against soft-rot and blue stain (Lohmann 2003). CuQuat - and Cu-triazoles preparations display a comparable efficacy range to Cu-HDO with regard to the target organisms (see above). Copper-triazoles named in the German index of wood preservatives (Immschler & Quitt 2005) contain propiconazole and/or tebuconazole (see below). All listed copper preparations are also available as boron-containing formulations.

After the ban of CCA formulations in the USA as from 2004, ammoniacal copper quat formulations (ACQ) serve as the primary substitutes. All ACQ types contain two active ingredients which may vary within the following limits: copper oxide (62%–71%), which is the primary fungicide and insecticide, and a quaternary ammonium compound (29%–38%), which provides additional fungicide and

insect resistance properties. The products contain either ammonia (type B) or ethanolamine (type D) as carrier solution. In formulate type C, ammonia and/or ethanolamine is used as the carrying solution; standardisation of type A was deleted in 2000 due to a lack of use (US Environmental Protection Agency 2005).

Further organic copper preparations are known, but play a minor role on the German market. These contain active ingredients such as Cu-naphthenate, Cu-citrate, Cu-bis(dimethyldithiocarbamate) (CDDC) und bis-Cu-8-quinolinolate (Oxin-Cu, Cu-8) (Schultz & Nicholas 2003).

Quaternary ammonium compounds (quats) are other chromate-free fixating preservatives. During treatment, quat compounds readily bind to the wood matrix; this hampers their penetration into deeper layers of wood and is an important shortcoming for the application (Richardson 1993).

The application of quat preparations is restricted to wood in the hazard classes 1, 2 and 3, since they do not exhibit fair efficacy in soil against soft rot (hazard class 4). In addition to a preventive application, quats are utilised to combat the dry rot *Serpula lacrymans* following the procedure described in the German standard DIN 68800-4 (1990).

The following quat preparations (P, Iv) are commonly applied for wood preservation:

- Didecyltrimethylammonium chloride (DDAC)
- Alkyldimethylbenzylammonium chlorides (benzalkonium chloride)
- Tertiary alkylamines (N, N-bis(3-aminopropyl)alkylamine)
- Alkyldimethylaminoxide
- Didecylmethylpoly(oxethyl)-ammonium propionate
- Didecylpolyoxethylammonium borate (polymeric betain).

Combining quats with boron compounds (quat-boron preparations) enables expanding the efficacy range of the preparation against wood destroying insects. It also enhances the leaching resistance of negatively charged boron ions, since these are bound to the positively charged ammonium ions in an ion pair complex (Kartal et al. 2005).

Boron compounds (P, Iv) do not fix in wood and remain easily leachable after treatment, even when chromium is added to the formulation. Since they are water soluble, they should be applied only under conditions, where leaching through weathering is minimised (hazard classes 1 and 2; Irmschler & Quitt 2005). The most common boron compounds are boric acid, sodium tetraborate (borax) or boric acid esters which are included in many water soluble/based preservatives systems. Boric acid and sodium tetraborate show a low solubility in water but much higher concentrations can be achieved by preparing a solution of 1 part boric acid and 1.54 parts sodium tetraborate decahydrate. Drying of this solution

yields a salt of the approximate composition of disodium octaborate tetrahydrate ($\text{Na}_2\text{B}_8\text{O}_{13}\cdot 4\text{H}_2\text{O}$) (Richardson 1993). Boron compounds show extremely low mammalian toxicity (Schultz & Nicholas 2003) and are partly available on the market as “biological” wood preservatives. However, they are toxic to plants when released into the soil (Wheeler & Power 1995).

Among the chromate-free fixating wood preservatives, the German index of wood preservatives (Irmschler & Quitt 2005) refers to a collective group (“Sammelgruppe”) which primarily comprises of organic biocides (see below). Within this group, quat compounds, propiconazole and the insecticide fenoxycarb are principally mentioned.

Organic solvent-borne preservatives

Solvent-borne preservatives are solutions of organic fungicides and/or insecticides in organic solvents (e.g. white spirit). In some cases, they contain other ingredients such as pigments, siccatives and water repellents (Lohmann 2003, Richardson 1993). In addition to pure organic agents, solvent-borne preservative systems may also contain e.g. Al-HDO or K-HDO (see above). Organic fungicides are generally active against wood-degrading and staining fungi, but not against soft rot fungi. Solvent-borne preservatives are suitable for dry wood and wood with a moisture content below fibre saturation point. The formulations are ready-to-use with a maximal active ingredient concentration of few percent and must not be diluted prior to application (Lohmann 2003). It is not possible to classify organic solvent borne preservatives similarly to water-borne preservatives because of manifold combinations of active ingredients resulting in a large variety of formulations.

The following active ingredients are utilised in solvent-borne and emulsion type preservatives (Richardson 1993, Irmschler & Quitt 2005, Lohmann 2003, Nicholas 2001, Schultz & Nicholas 2003):

Triazoles (P; against white/brown rot, blue stain fungi and moulds)

- Azaconazole (also against blue stain in service)
- Cyproconazole
- Propiconazole
- Tebuconazole
- TCMTB (2-(Thiocyanomethylthio)benzothiazole) (also against moulds and insects)

Phenylsulfamides (P; against blue stain fungi and moulds)

- Dichlofluanid (1,1-dichloro-N-[(dimethylamino)sulfonyl]-1-fluoro-N-phenyl-methanesulfenamide; DCFN)
- Tolyfluanid (also against blue stain in service)

Carbamates (P)

- IPBC, 3-Iodo-2-propynyl-butyl-carbamate (against white/brown rot and blue stain fungi)
- Carbendazim (Methylbenzimidazole-2-yl-carbamate) (against surface blue stain)

Aromatic fungicides (P)

- Pentachlorophenol (prohibited; against algae and fungi)
- Ortho-phenylphenol (against blue stain fungi and moulds)
- Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) (against white/brown rot fungi, bluestain, moulds and insects)

Organometallic compounds

- Al-HDO (Xyligen Al) (against white/brown rot fungi)
- K-HDO (Xyligen K) (against white/brown rot fungi)
- Tributyltin oxide (TBTO)
- Tributyltin benzoate (TBTB)
- Tributyltin naphthenate (all organotin compounds are effective against brown/white rot fungi, partly also against insects)

Further fungicides:

- Bethoxazin (against algae, brown/white rot, soft rot and blue stain fungi, moulds)
- 4,5-Dichloro-2-n-octyl-4-isothiazolin-3-one (Isothiazolon) (against brown/white rot, soft rot and blue stain fungi, termites)

Synthetic pyrethroids (Iv):

- Permethrin
- Cypermethrin
- Cyfluthrin
- Deltamethrin
- Silafluofen

Further insecticides

- Lindan (not prohibited, but hardly used anymore)
- Imidacloprid
- Flufenoxuron (Flurox)
- Chlorpyrifos
- Fenoxycarb (Farox)

Emulsions/micro-emulsions

Emulsions/micro-emulsions are mixtures of two or more immiscible liquids – also referred to as dispersions (solid compound solved in a liquid). In the case of wood preservation emulsions, water is the dispersing agent in which water insoluble organic biocides (disperse phase) are dispersed by means of emulsifiers. Aqueous emulsions increasingly replace organic solvents and can also be applied on wet wood. For the most part, emulsions are utilised for remedial wood preservation, particularly against insects, e.g. for large areas or bore-hole impregnation. In comparison with solvent-borne systems, emulsions show a poor penetration into wood due to the relatively high particle size of the dispersed agents (1-100 μm) – this is a main drawback of application of emulsions as preventive preservative. In contrast, micro-emulsions display a much lower particle size (0.01-0.1 μm) and, thus, penetration depths and velocities comparable with solvent-borne systems. Moreover, the colloidal stability is significantly increased compared to normal (macro-)emulsions. These properties make micro-emulsions suitable for preventive wood protection as a substitute for solvent-borne preservatives (Lohmann 2004).

Conclusions

According to a press release of the former Federal Institute for Health Protection, of Consumers and Veterinary Medicine (BgVV) from 21.9.2001, about 1500 wood preservative preparations were available on the German market at that time – many of them offered in do-it-yourself stores. However, less than one third of these products were evaluated or approved by independent organisations (cited in Zujest 2003).

The European Biocidal Product Directive 98/8/EG (1998) aims at establishing a “positive list” of approved active ingredients in order to guaranty high effectiveness and low impact on human health and environment. It was transformed into German law as Biocidal Law (Biozidgesetz) on June 20th 2002.

Wood preservatives approved by the DIBt or the Quality Assurance Association for Wood Preservatives always contain biocides to preserve wood against or to combat fungal or insect infection. The application of wood preservatives is dependent on defined hazard classes which differ in terms of moisture conditions and the predominant microbiological decay organisms.

The spectrum of wood preservatives ranges from tar oils, over a large variety of inorganic salts to complex organic compounds. Due to problems of waste wood disposal, the wood preservation industry aims at replacing inorganic salts by pure organic formulations, particularly for soil application, to enable incineration without toxic residues. In order to achieve this problem, organic biocides need to

be developed that are effective against soft rot fungi and that are concomitantly not detoxified by other soil organisms.

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14. Biological Wood Protection against Decay, Microbial Staining, Fungal Moulding and Insect Pests

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Introduction

Living trees in nature are likely attacked by various biological threats and pests, especially bacteria, fungi and insects (see also Chapter 11 of this book). Growth of trees might be affected and very aggressive challengers might kill trees before they reach the stage of chopping. Negative effects on wood quality include irregularities in the wood structure, unwanted staining, various forms and strength of decay (Fig. 1) and/or browsing damage. Thus, living trees may need protection when wanting the highest possible yield and wood quality. Furthermore, harvested wood

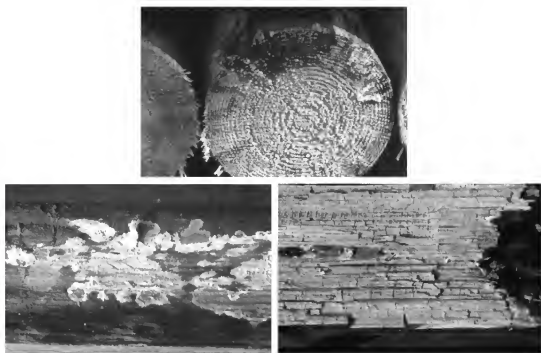


Fig. 1 Wood spoiled by sapstain (upper panel), white-rot (lower panel left) and brown rot (lower panel right). Photos were kindly supplied by U. Junga

is at risk to be harmed by mainly fungi and insects. Particular precaution is critical at harvest time and further handling depends on usage and environmental conditions at which wood and wood products are kept. Chemical methods of wood protection are powerful (see Chapter 13 of this book) but can bear certain environmental and health risks (for example see Westlund & Nohrstedt 2000, Dahlgren et al. 2003, Dube et al. 2004, Stook et al. 2005). Strategies for biological control of microbes and insects offer environmentally friendly wood protection of low health risks. **Biological control agents (BCAs)** are advantageous in that they are specific in targeting a biological hazard, they are readily biodegradable and they might become a persistent component of ecosystems eliminating a need of repeated treatments. However, compared to chemical agents, BCAs might be more sensitive to climatic conditions and adequate handling requires specific knowledge. Since mostly living material, BCAs have a limited shelf life but this problem might be corrected by improving formulations. Most importantly, unwanted shifts in the biodiversity of ecosystems by introduction of BCAs have to be avoided. This requires extensive research on ecological effects before licensing an agent for a specific environment – preferentially, the BCA should be of indigenous origin (for further discussion see Headrick & Goeden 2001, Lacey et al. 2001, Shah & Pell 2003). In living tree and in wood protection, some biological control products of harmful fungi and insects have reached commercial

production and application (van Frankenhuyzen et al. 2000, Pratt et al. 2000, Lacey et al. 2001, Potter & Held 2002). In other cases, research to obtain ecological and economical viable products is actively ongoing (e.g. Phillips-Laing et al. 2003, Chapman et al. 2004, Wright et al. 2004) but often, suitable control systems have still to be found, developed and/or extensively tested.

Biological control in protection of standing trees

Heterobasidion - Successful biological control in nature

Within the Northern temperate forests, *Heterobasidion annosum* and allies represent the most important disease of living conifers by causing root and heart rot through degradation of lignin and cellulose components of the wood. Yearly losses in Europe are estimated € 7.9 x 10⁸. The fungus infects healthy tree stands primarily by basidiospores which are produced abundantly within the fruiting bodies and distributed by airflow up to several hundreds of kilometres. The spores germinate on and invade exposed wood surfaces of stumps or wounds on roots and stems. In secondary infection, the fungus distributes further from tree to tree by mycelial growth along roots. Vegetative spread within stands produces expanding disease centres before it eventually stops after some decades (Woodward et al. 1998, Asiegbu et al. 2005). Avoiding primary infection in healthy stands is therefore the target for biological control of *Heterobasidion*. Following early observations on strong antagonistic effects of the saprophytic white rot fungus *Plebeiospora gigantea* towards *H. annosum* in colonising freshly-cut conifer stumps (Rishbeth 1963), the fungus has been applied for decades to stumps in the field for prevention of *Heterobasidion* infections (Pratt et al. 2000). Throughout Europe, *P. gigantea* stump treatment is practised on more than 200,000 ha annually (Thor 2003). For application on the stumps, during or immediately after felling asexual spores (oidia) are spread in high densities (ca. 200-1000 spores/cm²) in mechanical or hand-held application in order to quickly germinate and invade the substrate, thereby eliminating the chance for *Heterobasidion* to occupy the same (Gibbs et al. 2002, Thor & Stenlid 2005). Thus, the main principle of antagonism resides in competition for the wooden substrate. In addition, hyphal interference, negative effects by *P. gigantea* on hyphal structures of *Heterobasidion* up to causing their death, has also been observed (Ikediugwu 1976).

In 1998, the PG Suspension, *P. gigantea* oidia in sucrose solution, was given full approval for use as a fungicide for *Heterobasidion* control under UK Pesticides regulations 1986 (Pratt et al. 1999). PG IBL is a market product approved for usage in Poland and Rotstop for usage in Scandinavia and Switzerland. These products distinguish in formulation - the first presents oidia in sawdust and the second in a wettable silica powder. All three commercial products are active on pine, Rotstop in addition on Norway spruce stumps. The strains of the products originate from the UK, Poland and Finland, respectively. Only Rotstop is a pro-

duct of a single isolate (Pratt et al. 2000). Recent reports of reduced performance of this Finnish strain in Sweden lead in year 2004 to the formulation of a Rotstop[®] variant with an effective Swedish isolate (Berglund et al. 2005). Tests with the different Rotstop variations, however, did not reveal differences in infection strength over a broader range of spore concentrations (Ronnberg et al. 2006). For effective protection, care has to be taken of complete stump covering (Nicolotti & Gonthier 2005, Ronnberg et al. 2006).

Application of *P. gigantea* isolates in their native countries appears to influence negligibly the genetic diversities of local populations. Mycelial spread of *P. gigantea* from stumps into the neighbourhood is poor. Six years after application, the fungus is rarely detected any longer in treated stumps and after this time, persistence of fungal species other than *Heterobasidion* within the stumps appears little affected (Vainio et al. 2001, 2005, Vasilikauskas et al. 2005). *P. gigantea* is a common coloniser of fresh conifer wood in boreal and temperate forests throughout the world. In Europe, *P. gigantea* is regarded to be a species with a low level of regional genetic differentiation. Lack of country-specific genetic markers implies that introduction of the marketed strains into other local European populations should not be a threat on genetic diversity of the species (Vainio et al. 1998). Nevertheless, effects of hybridisation between local and introduced strains on ecology can not be foreseen and usage of local isolates is preferable, or as in case of natural reserves, even mandatory. Accordingly, a native strain has been isolated from an Italian natural reserve at risk and shown to be as effective in protection of Italian stone pine as the Finnish Rotstop (Annesi et al. 2005). European and North American isolates are interfertile suggesting they belong to a single species, but some genetic differentiation including variations in the ITS (internal transcribed spacer) region has been encountered (Vainio & Hantula 2000, Grillo et al. 2005). Formulations of own isolates have been applied in the USA for several years, but currently there is no state approval in North America for the use of *P. gigantea* as a pesticide (Ross & Hodges 1981, Pratt et al. 2000).

A cost range comparable to chemical treatments, uncomplicated manual or mechanical application and the ease of formulation by abundant oidia production are main reasons for the commercial success of *P. gigantea* as *Heterobasidion* BCA in European countries (Pratt et al. 2000). However, the restricted range of conifer species being protected by strains of *P. gigantea* requests identification of further BCAs for other tree species. Many other micro-organisms, mostly fungi but also bacteria, have been shown to be antagonistic to *Heterobasidion* based on competition for resources (e.g. the white rot fungus *Resinicium bicolor*), production of antibiotics, toxins, or fungal cell wall degrading enzymes (e.g. the brown rot fungus *Fomitopsis pinicola* and the ascomycete *Trichoderma harzianum* and the hyphomycete *Phaeothonia dimorphospora*) and/or mycoparasitism (e.g. *Trichoderma polysporum*). In some cases, the capacity of antagonism is strongly influenced by environmental conditions such as the prevailing temperature or pH. Some organisms, for exam-

ple the hyphomycete *Scytalidium lignicola*, show remarkable antagonistic activity in *in vitro* tests on artificial medium but have little effect in wood tests and/or under natural conditions in stump infection trials. In contrast, *R. bicolor* has been reported to be little active against *Heterobasidion* on artificial medium but to be most competitive on wood. Identification of potential antagonists by simple laboratory methods therefore needs to be strengthened by evaluation under natural conditions, also under the aspect of not disturbing the natural ecosystem. Further on, emphasis has to be given to production and formulation of the BCA. *R. bicolor* for example produces no spores *in vitro*. Therefore, a pertinent *R. bicolor* formulation could involve fragmentation of mycelium. Crushing to small fragments however reduces viability dramatically. Burying infected wood blocks besides stumps, dowel inoculation of stems and stumps and colonised sawdust have been tested with so far not satisfying results (Holdenrieder & Craig 1998, Murray & Woodward 2003, Roy et al. 2003, Berglund et al. 2005, Woods et al. 2005).

Biological control of other pathogenic fungi

Many other fungal species affect living trees by white rot, brown rot, soft rot, shoot and bark diseases, or leaf diseases and defoliation (Schwarze et al. 2000, Agrios 2005). Biological control schemes are desired but so far mostly lacking. The current progress in biological control of the aggressive tree pathogens *Armillaria* and *Ophiostoma* is presented in the following. Various *Armillaria* species are serious root pathogens that worldwide cause root and butt rot on conifers as well as on hardwoods (Shaw et al. 1991). Areas of hundreds of hectares can be infected by one single isolate with devastating killing effects on the trees (Ferguson et al. 2003). Some reports are available on potential BCAs, including wood-decay fungi with similar ecological niches (Boddy 1993, Pearce et al. 1995, Chapman & Xiao 2000, Chapman et al. 2003) and toxin producing and mycoparasitic *Trichoderma* species and other ascomycetes (Dumas & Boyonowski 1992, Reaves & Crawford 1994, Otieno et al. 2003, Raziq & Fox 2004). Burying the mycoparasite *T. harzianum* after growth on grains or other plant material in the soil in close vicinity to infected roots appears to be a promising strategy of killing *Armillaria* hyphae and rhizomorphs (Raynal et al. 2002, Otieno et al. 2003). *P. dimorphospora*, originally isolated as a fungal antagonist of the ascomycete *Ophiostoma ulmi*, inhibits by organic fungicidal and fungistatic compounds a broad range of fungi damaging roots, wood, bark and foliates of trees, including *Armillaria* and *Heterobasidion* (Yang et al. 1993, 1994; see above). Conditioning inoculation of seedlings with *P. dimorphospora* protected *Ulmus americana* against its pathogen *O. ulmi* but unfortunately not against the more aggressive agent of Dutch elm disease, *Ophiostoma novo-ulmi* (Bernier et al. 1996). Probably via triggering the host defences, conditioning inoculation with a certain isolate of the vascular wilt-fungus *Verticillium dahliae* significantly reduced wilt symptoms by *O. novo-ulmi* and the isolate has been used to preven-

tively treat more than 160 000 elms in the Netherlands and in the USA. However, another *V. dahliae* strain caused wilt symptoms in elms themselves and, in addition, bark beetles were strongly attracted to the trees, indicating a danger in the strategy of preventive use of fungal pathogens from other sources in fighting diseases of other plant species. Conditioning inoculation using *Pseudomonas* spp., other fungi nonpathogenic to elms or also *O. ulmi* as antagonists against the aggressive *O. novo-ulmi* has been tested with variable success (Solla & Gil 2003 and references therein).

Biological control of harmful insects

Fungal defoliation and bark diseases are often distributed via insect vectors - for example, elm bark beetles transfer *Ophiostoma*. Next to fighting the fungus directly, attempts to control the disease target on reduction of insect vector populations (Hubbes 1999). Fungi infectious to the beetle's larvae exist (Houle et al. 1987) but have so far not given rise to a biological control programme. Principally, insects might be harassed at all developmental stages. Fighting juvenile stages with defined accessible territories can be advantageous over combating imagos, particularly if these are short-lived and mobile (Shimazu & Sato 2003).

In addition to being vectors, various insects cause direct damage by feeding on the plant material. Examples for defoliators able to kill trees are the gypsy moth *Lymantria dispar* feeding on leaves from oaks and aspens, *Choristoneura* spruce budworms, *Neodiprion* sawflies and the pine beauty moth *Panolis flammea* living from conifer needles and the hemlock looper *Lambdina fiscellaria* whose early developmental stages feed predominantly on new shoots, older ones on the total foliage (van Frankenhuijzen et al. 2000). Classical biological insect control programs in forests are based on spray application of *Bacillus thuringiensis* and its ingestion together with endotoxins (Bt toxins) produced by the bacterium (Cibulsky et al. 1993, Evans 1997, van Frankenhuijzen et al. 2000, Bauce et al. 2004). Within the insects, the toxins are believed to destruct transmembrane potentials resulting in osmotic lysis of cells lining the insect's midguts (Aronson & Shai 2001, Whalon & Wingerd 2003). With the identification of the very effective *keurstakei* variety of *B. thuringiensis* (*Btk*), optimisation of production, formulation and plane spraying techniques and the political shift in favour of biological products, application of *Btk* is cost-effective since the mid 1980s. For certain forest pests in Canada and USA, *Btk* is now the only agent in use (van Frankenhuijzen et al. 2000), in Eastern Europe application is constantly increasing (Evans 1997). An eradication programme with *Btk* in Auckland's eastern suburbs against the white-spotted tussock moth *Orgyia thyellina*, a serious forest defoliator imported from Asia, was the first worldwide reported to have fully erased the targeted pest in an urban area (Hosking et al. 2003).

Freely spraying of *Btk* formulations into the environment is however not without any ecological risks. Monitoring in forests in British Columbia treated with *Btk* to control the western spruce budworm, for example, revealed negative effects also on nontarget Lepidoptera (Boulton et al. 2002, Boulton 2004, Boulton & Otvos 2004). To exclude unwanted effects by freely spraying on uninvolved insects, newer developments make use of the bacterial endotoxin genes. When expressing such genes in transgenic trees, only insects eating leaves or needles are challenged (see Chapter 7 of this book for further information on transgenic trees). First results on transgenic *Picea glauca* indicated that the needles of white spruce became lethal to spruce budworm larvae by expression of the endotoxin (Lachance et al. 2007). Transgenic *Populus nigra* observed during 1994–1997 in China had only 10% of leaves highly damaged by defoliators compared to 80–90% of leaves of non-transformed trees in neighboured control plantations. Values of pupae in soil were reduced to 20% far below the threshold set for chemical protection. Furthermore by reducing the total number of insects in the environment, cross protection of non-transformed trees occurs by transgenic trees in the same plantation (Hu et al. 2001). In a field trial in the USA, transgenic hybrid *Populus* also showed improved protection against defoliators by endotoxin expression, together with an increased mortality of early developmental stages of the insects (Kleiner et al. 1995). A recent study on decomposing cotton tissue in soil revealed a long Bt persistence in the environment. The Bt half-life was at least 56 days (Sun et al. 2007). Similarly, Bt from transgenic rice can remain such long or even longer periods in the soil – depending on the prevailing conditions such as pH and water content (Wang et al. 2007). Such results point out a potential new environmental problem – arthropod populations in soil might be affected by the decaying material from transgenic plants (Torres & Ruberson 2007). Moreover of concern for application of BT transgenes is the possible acquisition of resistances by the pests. For example, resistant *Chrysomela tremulae* beetles were found feeding on transgenic Bt poplar with a mortality similar to that of beetles fed with non-transgenic poplar leaves. Such findings call for implementation of suitable resistance-management strategies together with any release of transgenic resistant trees into nature (Génissel et al. 2003, Augustin et al. 2004). Generally, effects of transgenic trees on ecosystems have carefully to be evaluated (van Frankenhuijzen & Breadmore 2004; see Chapter 7 of this book). If concerns about release of genetic engineered trees can be ruled out, broader application of transgenic trees is most likely limited to forest plantations of fast growing species (Fenning & Gershenzon 2002).

Baculoviruses are other alternatives of controlling insect pests, independent of the tree species attacked. They are double-stranded DNA viruses which are highly selective for several insect groups. After ingestion by the host, the viral DNA is released by dissolution of the viral capsids and enters the nuclei of the midgut epithelial cells, where it replicates and from which it spreads into other tissues

within the host (Lacey et al. 2001). Compared to *B. thuringiensis*, spreading of baculoviruses has ecological advantages through their high degree of host-specificity and their efficient horizontal and vertical transmission within a species, i.e. within and between generations. Furthermore, baculoviruses are amenable to recombinant DNA technology enabling deletion of genes that delay host mortality or insertion of others promoting fast uptake and/or mortality (van Frankenhuijzen et al. 2000, Inceoglu et al. 2001, Lacey et al. 2001). Neocheck-S® and Virox® are American and British baculovirus products against the sawfly *Neodiprion sertifer*, Lecontivirox® a Canadian product against *Neodiprion lecontei*. TM Nio-Control-1® and Virtuss® act against the Douglas-fir tussock moth and are registered by the USDA (United States Department of Agriculture) and the Canadian Forest Services, respectively. Despite good success in technical handling and excellent results in field tests, the products are either not in use, or if, only on a small scale. Since limited introduction into pest populations at one time is sufficient for control without the need for further interventions, commercialisation is discouraged (van Frankenhuijzen et al. 2000, Williams & Orvos 2005).

One principle control strategy is to permanently regulate a pest after initial introduction of a BCA by circulating with episodic populations. Such a sustainable scheme is established in control of *L. dispar* by the fungus *Entomophaga maimaiga*. This entomopathogenic zygomycete established serendipitously in the early 1900's in Northern-East parts of the USA, infects and grows within gypsy moth and now spreads further by wind distribution of spores and human intervention over the country, thereby helping to eliminate the insect problem (Lacey et al. 2001, Gillock & Hain 2002, Shah & Pell 2003). Infection by *E. maimaiga* appears to be negatively influenced by dry conditions, possibly by low production of the infectious spores. Under such unfavourable conditions, application of *Btk* and gypsy moth specific baculovirus can support mortality of the insects (Mott & Smitley 2000). Watering plots may be another measure in *E. maimaiga* management (Webb et al. 2004). A second example of classical biocontrol by newly introducing an antagonist is *Paenibacillus* (*Bacillus*) *popilliae*. In 1950, this bacterium was the first registered microbial control agent in the USA. It causes milky disease of the larvae of the Japanese beetle, *Popillia japonica*, an invader to the country in around 1900 whose larvae feed on roots of grass and the adults on the foliage of broad-leaf trees. Unfortunately, the bacterium only proliferates in the living host, i.e. within the beetle's larvae that adopt a white appearance when fully filled with the bacteria (therefore the name milky disease). *P. popilliae* products are available on the market, but dependence on *in vivo* growth in larvae collected from the wild limits commercial mass production (Klein & Kaya 1995, Potter & Held 2002).

For rapid short-term control of insect pests without expectation of secondary waves of infection, formulations of entomopathogenic fungi (mycoinsecticides) may be applied repeatedly and in often large amounts. *Beauveria* Schweizer is a Swiss product of the hyphomycete *Beauveria brongniartii* grown on barley kernels

(Enkerli et al. 2004, Kessler et al. 2004) that was developed to specifically control the European cockchafer *Melolontha melolontha*, a sporadic pest feeding on foliate, flowers and fruits as an adult and on grass plant and tree roots as a larva. Other well known, but less specific mycoinsecticides used in repeated control of various insects are the hyphomycetes *Beauveria bassiana* and *Metarhizium anisopliae* (for further details on fungal control of insect pests see Shah & Pell 2003). Genetic markers [microsatellites, randomly amplified polymorphic DNA (RAPD) markers, restriction fragment length polymorphism (RFLP); for more detailed explanations see Chapter 8 of this book] have been defined to monitor *Beauveria* and *Metarhizium* strains in the field (Leal et al. 1994, Maurer et al. 1997, Bidochka et al. 2001, Enkerli et al. 2001, 2005).

Biological control after felling

Following cutting and during the several month-lasting period of drying and storage in forests and sawmill yards, the timber is specifically susceptible to decay and microbial staining until the water content within the wood decreases to about or below 25%. Felling during the winter and suitable storage helps to prevent microbial infection, in particular blue stain and mould, that easily occurs under very humid climates and poor aeration. Furthermore, some progress has been made in establishing biological measures in postharvest control of unwanted surface discolouration sap-wood staining and decay (Mai et al. 2004, Yang 2005).

Laboratory screening programs lead to long lists of microbes reported to have antagonistic effects to various kinds of decay and stain fungi. However, in subsequent tests in outer conditions few of the organisms tend to be reliable in wood protection (Graf 1990, 2001). Application of suspensions of 10^6 spores/ml of *Glucidium roseum* for example provided satisfactory protection against colonisation of various moulds and sapstain fungi in wood wafer tests of western hemlock, white spruce, jack pine, amabilis and balsam fir but not of Douglas fir, white birch and trembling aspen (Yang & Rossignol 1999). These promising results lead to the development of a new U.S. patented bioprotectant in form of an albino *G. roseum* powder product. Following 11 month storage in a lumberyard, 90% of black spruce and balsam fir lumber treated with the bioprotectant did not get any unwanted fungal infection compared to 5% of untreated wood. Concerning mould and sapstain, 100% of the treated wood was acceptable for marketing but only 16% of the control boards and only 0.7 % of treated boards were seriously affected by decay fungi compared to 45 % of untreated boards (Yang et al. 2004).

Other good candidates for efficient biocontrol measures during kiln-drying and short-term storage at sawmills are some single-celled, easy to produce and formulate yeasts and bacteria that produce a range of volatiles causing growth inhibition of sapstain fungi (Bruce et al. 2003, 2004). Treatment of wood with these microbes before, during or even after infection by the target fungi was shown to pre-

vent wood spoilage. Under laboratory conditions, visual spoilage of wood blocks of *Pinus sylvestris* by mould and sapwood staining was reduced by the bacterial isolates, due to the production of the antifungal volatiles. *Debaryomyces* yeast strains protected wood blocks under conditions of high relative humidity and a variety of temperatures between 15 to 25  C at a spraying rate of 10^8 colony-forming units (CFU)/cm² against a mixture of wood-mould fungi and at a spraying rate of 10^6 – 10^8 CFU/cm² against sapstain fungi (Payne et al. 2000, Payne & Bruce 2001).

Microbial sapwood staining is mostly caused by the ascomycetous yeast *Aureobasidium pullulans* and various filamentous ascomycetes and related hyphomycetes belonging to *Ophiostoma*, *Ceratocystis*, *Cladosporium*, *Alternaria* and other genera (Yang & Rossignol 1999, Fleet et al. 2001). These fungi live on sugars, lipids and resins present as storage and protective materials within ray parenchyma and resin canals of the sapwood (Martinez-Inigo et al. 1999, Fleet et al. 2001). Usually, they cause little or no destruction and strength loss to the wood (Blanchette et al. 1992) but the unsightly staining represents loss of value for production of solid wooden objects as well as for paper and cardboard production (Vanneste et al. 2002). Most sapstain is within the grey-blue-brown-black range (commonly referred to as blue-stain) and comes from melanin that is incorporated in the cell walls of fungi colonising the ray parenchyma and shines through the wood (Brisson et al. 1996). Pink wood staining by *Arthragraphis cuboidea* is caused by secretion of naphthoquinone pigments (Golinski et al. 1995) and green wood staining in the case of *Chlorociboria* species by secretion of complex quinones (Saikawa et al. 2000). As indicated by the examples discussed above, sapstain infection might be inhibited by colonisation of the sapwood by other micro-organisms competing for the nutrient source or by suitable metabolites preventing fungal growth. *Trichoderma* species have been shown to control sapstain fungi by both modes (Vanneste et al. 2002). A problem with potential fungal control agents competing for the sapwood nutrients however is that they themselves may disfigure the timber by own pigments (Smouse et al. 1999, Payne et al. 2000). Agar screening tests showed that the yeast *Galactomyces geotrichum* produces enzymes that might help to remove the fungal melanin from wood (Ratto et al. 2001). As shown in both laboratory and field trials, the white rot fungus *P. gigantea* in contrast is able to decolourise previously stained sapwood by parasitising on hyphae of bluestain fungi, in addition to effectively inhibit *Ophiostoma* species in wood colonisation (Behrendt & Blanchette 2001).

The most elegant biological solution to deal with the sapstain problem is the inoculation of freshly sawn timber by colourless mutants of sapstain fungi that lack any melanin (Behrendt et al. 1995, Held et al. 2003). The U.S. product Cartapip 97   is a market formulation of such an albino *Ophiostoma piliferum* mutant that was originally designed as a pitch control agent of wood chips prior to pulping. As a side effect, treated wood chips showed better resistance to infection of other micro-organisms including sapstain fungi (Blanchette et al. 1992, Farrell et al. 1993, Wang et al. 1997, Dorado et al. 2000). This prompted to apply the

mutant in the laboratory or in fields on freshly sawn wood before challenging with other sapstain fungi. Protection was fully given (up to 100%) by inhibition of wood colonisation by other fungi. Some protection was still encountered when simultaneously applied with other fungi, but Cartapip 97® had no effect once the wood was colonised by other strains (Behrendt et al. 1995, White-McDougall et al. 1998). In 2004, under the new name Sylvanex Technical, a wettable powder formulation of the albino *O. piliferum* strain D97 has been granted temporary registration under the Canadian Pest Control Products Regulations for the use on freshly felled lodgepole pine and red pine at the felling sites only (Pest Management Regulation Agency, Ontario 2004). Biological field trials of Cartapip 97® were carried out in other countries (New Zealand, Germany, England) under their special legislation and control conditions. However, before registration as BCA by different states the ecological safety has to be evaluated. To follow up the fungus in field-tested logs in Germany, specific genetic markers (the gene coding for β -tubulin together with two sequence-specific primers, Cat1 and Cat2) have been defined that distinguish the strain from resident strains. The marker also appears to be useful for other European countries, New Zealand, Alberta and British Columbia but it has limitations in monitoring the mutant in certain regions of the USA and Canada due to lack of distinction from wild strains (Schröder et al. 2002). Cartapip97® strain is not a virulent pathogen to local pines in South Africa and therefore has been judged as safe for use (Dunn et al. 2002). However, *O. piliferum* was never found in South Africa and quarantine restrictions of the state block the import of the BCA. Related species exist that instead may serve in isolating albino mutants for control of sapstain fungi in the future (de Beer et al. 2003). A spontaneous, Kaspar named mutant from *Ceratocystis resinifera* has recently been tested in Canada as an alternative to Cartapip97®. It had higher protection rates (up to 94.4% in the laboratory, 80% in the field) than the established biocontrol agent (Morin et al. 2006).

Biological control of timber in service

Microbial and insect threats to wood in service very much depend on the type of application, whether indoors or outdoors, and the use classes (EN335-1, see Chapter 13 of this book). Wood with a water content of 12% or below, as typically found within buildings, is not susceptible to biological hazards except insects. Wood moisture of 12-18% facilitates attack by insects. Moulds and staining fungi can infest wood when moisture content rises (sporadically) above 18%, as for example observed indoors in humid-rooms and outdoors for sheltered wood. Decay by brown- and white-rot basidiomycetes requires a moisture content above the fibre saturation point (about 28-33%). Beyond this point, unbound water is found in cell lumina. Permanently wet wood in soil contact is vulnerable to fungi (soft-rot) and bacteria (Huckfeldt et al. 2005; see Chapter 13 of this book).

Biological control of adverse fungi

Humidity, temperature and ventilation very much influence fungal prevalence and microbial biodeterioration within buildings. Amongst many other decay fungi, the dry rot fungus *Serpula lacrymans* is found uniquely destructive in buildings under poor climatic conditions (Huckfeldt et al. 2005). Various *Trichoderma* strains were tested as potential BCAs against this economically most important brown-rot fungus. Regardless of whether viable or non-viable, some *Trichoderma* isolates were shown to prevent *Serpula* colonisation in laboratory wood block tests, but they did not stop decay of already infected blocks (Score et al. 1998). Strains were found to be also active in killing *Serpula* in the absence of direct contact, indicating that the mode of antagonism employs inhibitory volatile organic compounds (VOCs) (Humphris et al. 2002, Phillips-Laing et al. 2003). Various volatiles are produced by *Trichoderma* species (e.g. heptanal, octanal, nonanal, decanal and related ketones) and shown to react on a range of brown- and white-rot fungi including *Trametes versicolor*, *Neolentinus lepideus*, *Postia placenta* and *Gloeophyllum trabeum*. In a field and cellar trial setup, the most promising *Trichoderma viride* strain T60 had a protective effect against decay, soft rot and sapstain fungi (Brown & Bruce 1999, Brown et al. 2000, Humphris et al. 2001; for further information on fungal VOCs see Chapter 11 of this book). Production of VOCs by *Trichoderma* is determined by cultural age and media composition, mainly by the amino acids available and less by the sugars. Therefore, the amino acid content present in timber to be protected likely plays a crucial role on the effectiveness of antagonism by *Trichoderma*. Because of the limitations of nutrients in wood for the fungus, protection by volatiles is expected to last for a certain time, whilst persistence in wood protection by *Trichoderma* is likely not achievable (Canessa & Morrell 1997, Bruce et al. 2000). Production of extracellular proteases is an additional possible measure of antagonism by mycoparasitic *Trichoderma* strains (Skezeres et al. 2004). *Trichoderma* strains can act also bactericidal by production of bacteriolytic enzymes (Manczinger et al. 2002), but effects on growth of bacteria in wood have not yet been evaluated. The kind of usage and the general water content of the timber will likely influence the economical success of preventive *Trichoderma* application (Kredics et al. 2003, 2004). Application of the commercial agent BINAB FYT® (pellets of spores and mycelial fragments of *T. polyosporum*, *T. harzianum* und *Scytalidium* spp. FY) to impregnated distribution poles under environmentally demanding outdoors conditions gave variable results. *N. lepideus* and *Antrodia carbonica* were inhibited even after seven years whereas protection of the pole interiors against by *T. versicolor* failed (Bruce et al. 1991).

Biological control of insect pests

Efforts to develop efficient BCAs against the various kinds of insects attacking timber in service (e.g. house longhorn, ants, anobiidae beetles) focus on gluttonous subterranean termites, some of which also have the propensity to destroy

living wood. Alone in the USA, losses due to termite damage and control is estimated to cost U.S. \$ 1-2x10⁹ annually (Culliney & Grace 2000, Rath 2000, Lax & Osbrink 2003).

The entomopathogenic hyphomycetes *B. bassiana* and *M. anisopliae* are most promising in biological termite control (Culliney & Grace 2000, Le Bayon et al. 2000, Wright et al. 2004, 2005) but pathogenic bacteria such as *Serratia marcescens* strains causing death upon ingestion and infectious nematodes are also available (Graf 1990, Connick et al. 2001, Osbrink et al. 2001, Grace 2003, Mankowski et al. 2005). *Metarhizium* isolates most virulent towards termites are from termite-associated materials (Milner 2003, Wang & Powell 2004, Dong et al. 2007) whereas *Beauveria* strains from termites and from other sources are equally efficient (Wang & Powell 2003). Treatments for population suppression might be targeted either on the whole colony or on individual foraging workers that subsequently transfer the biocide to their nestmates (Rath 2000). In the laboratory, both fungal species need about 24 h from spore application to germination and insect penetration and subsequently about 48 to 72 h for killing the insects. Sporulation from the cadavers follows for the next 3 to 4 days (Neves & Alves 2004). Apparently, sporulation plays a role in fungal prevalence in termite populations and in possible outbreaks of epizootics (Sun et al. 2003). In Petri dish experiments, spores of the mycoinsecticides were transferred by infected workers to healthy individuals and totally mortality reached 50-100% within a few weeks (Le Bayon et al. 2000, Wright et al. 2002, Wand & Powell 2003). Marked differences were observed depending on whether individual insects or groups are treated. Fungal spores spread onto groups of termites were removed by mutual grooming within a few hours in contrast to spores applied on individuals (Shimizu & Yamaji 2003, Meikle et al. 2005, Yanagawa & Shimizu 2007). Termites show strong alarm and defence responses towards spore-dusted fellows (rapid bursts of longitudinal oscillatory movement, aggregation of untreated insects, grooming, biting, defecation and burial of infected termites). For successful fungal control in the field, such social behaviour needs to be overcome by strategies such as masking the repellency of spores, addition of attractants, definition of dosage levels below alarm thresholds and selection of less detectable strains (Staples & Milner 2000, Myles 2002, Milner 2003, Meikle et al. 2005). Formulations of *M. anisopliae* with attapulgitic clay and surfactant and *M. anisopliae* treated cellulose baits were reported to overcome repellent effects (Rath & Tidbury 1996, Wang & Powell 2004).

Conclusions and future perspectives

In the year 2004, we published a review on biotechnology in the wood industry including biological control of fungi and insect pests in timber from felling to in service (Mai et al. 2004). This paper updates the impressive advancements made since then and, in addition, it includes biocontrol of living trees. In the forests, a

number of commercial biocontrol products have been in use for years and others against various threats of living trees are arising. Now, in Canada, there is also the first registered biocontrol measure for felled conifer logs. For successful implementation of biocontrol agents, the ideal organisms have to be found and efficient, storable formulations and optimal application conditions to be defined. With an increasing number of biocontrol agents, understanding their mechanisms and increasing experience on handling, product development for other threats promises to become easier.

Long-term effects of application of biocontrol agents on ecosystems remain a major concern to be clarified. As seen by the examples in the text, this can be followed by defining natural strain-specific DNA markers. In addition, there is also the possibility to mark a strain by DNA transformation. To understand wood colonisation and challenging sapstain fungi, the Cartapip 97® white *Opbiostoma* mutant strain was transformed with the green fluorescent protein (GFP) gene *gfp* of the jellyfish *Aequorea victoria* and the transformants were monitored within wood by *gfp* expression (Lee et al. 2002). In other cases, DNA transformation has been used to enhance fungal properties in challenging the target organism. Genetic engineered *M. anisopliae* clones carrying the *gfp* gene alone or in combination with extra copies of the fungal protease gene *prt1* were monitored in a field study in Maryland. The recombinant fungi were found genetically stable over one year, dissemination was little and negative effects on the indigenous fungal microflora and non-target insects were not encountered (Hu & St. Leger 2002). The extra *prt1* copy numbers in the transgenic strain lead to enhanced secretion of the cuticle-degrading protease and to a 25% reduction of time of death and 40% reduced food consumption of the moth *Manduca sexta* (St. Leger et al. 1996). Similarly, recombinant overexpression of the chitinase gene *Bbcht1* in *B. bassiana* enhanced insect virulence by reducing lethal concentrations and lethal times by 50% (Fang et al. 2005). Whilst these results show great potential, they again address the principle problem of whether to make use of genetically engineered organisms.

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Part V – Panel Boards and Biotechnology

15. Panel Boards and Conventional Adhesives

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Introduction

For millennia, the natural dimensions of trees have defined the parameters of constructions made by humans. However, the naturally grown raw material wood varies in its chemical and physical properties depending on the timber species and their places and conditions of growth. Solid wood in its natural state has the significant disadvantage that relevant strength properties exist only in one dimension, i.e. longitudinal to the fibre direction. The strength across the fibre is only about 1/20 to 1/10 of the strength in the longitudinal direction (Nowak & Drach 1949).

Characteristically, wood shows a strong moisture-induced swelling and shrinking anisotropy. The relative proportions in tangential-radial-axial swelling of wood are about 20 to 10 to 1 (Kollman & Cote 1968, Skaar 1988, Chauhan & Aggarwal 2003, Sonderegger & Niemz 2006).

The term **panel board** - or **wood composite** - denotes any product, which is manufactured on the basis of mechanically chopped, milled and grinded (and refined) wood [veneers, strands, particles, fibres, etc. (Marra 1972)] and bonded by adhesives usually by an operation at high temperature and pressure (Malony 1993, Youngquist et al. 1997, Kharazipour 2004; Fig. 1). When producing panel boards, the homogenised raw material can be formed in any dimension and amount.

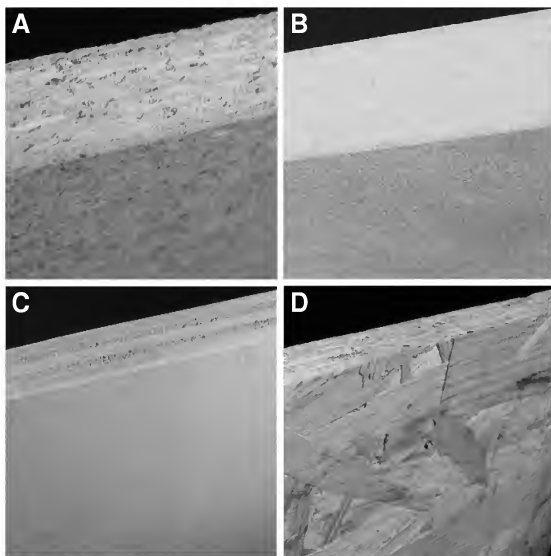


Fig. 1 The most important types of panel boards. A. Particle board (PB). B. Medium density fibreboard (MDF). C. Plywood. D. Oriented strand board (OSB)

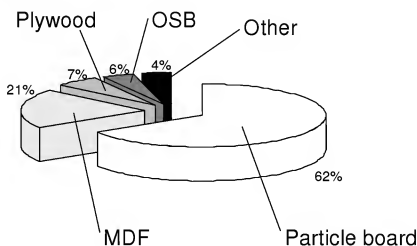


Fig. 2 Panel board production in Europe in the year 2005 (according to EPF 2006 and Marutzky 2006; see also VHI 2007a)

During the transformation into plane panel boards, the major problems of solid wood become ineffective. Due to the gluing of wooden material in different fibre directions, the dimensional stability in panel boards defeats that of compact wood in strength and by a better balance. In addition, panel boards are characterised by partly better static properties and reduced swelling-shrinking anisotropies (see for example Niemz & Poble 1996, Wu 1999, Thomas 2001, Lee & Wu 2002, Miyamoto et al. 2002, Sonderegger & Niemz 2006).

The availability of high quality sawnwood is generally limited. Depletion of forests, particularly also the tropical forests, to meet the demand for high-quality construction material has devastating ecological consequences (see Chapters 2 to 5 in this book). Another very important reason for the production of wood composites is therefore to save natural resources for sustainability. By panel board production, wood types with properties less favourable for application in compact form, low grade wood material from forests (e.g. from thinning in young forests, wastes from felling), left over parts from the timber industry and used wood products discarded for recycling can be made use of in order to convert these into a highly valuable product with excellent properties (for details on recycling see Chapter 20 of this book).

The most important panel boards are **particle boards**, **medium-density fibreboards (MDF)**, **oriented strand boards (OSB)** and **plywood** (Youngquist et al. 1997, Kharazipour 2004; Fig. 1 and 2). A particle board is defined as a wood-based panel manufactured under pressure and heat from particles of wood (wood chips) usually under the addition of an adhesive (EPF 2005). MDF is made from lignocellulosic fibres combined with a synthetic resin in a dry process by hot-pressing (Bolton & Humphrey 1988, Thoemen & Humphrey 2003). OSB is produced

from wood shredded into rectangular strips of a few centimetres in length, by layering these strands across each other in the direction of their grain, pressing, and binding them with resin adhesives (Brinkmann 1979, Lee et al. 2006). Plywood as the first engineered wood invented is made from sheets of wood veneer stacked onto each other, crosswise to the directions of their grains (O'Halloran 1989).

History of particle boards

Already around 2000 B.C., people in Egypt knew how to produce plywood from thin wooden slats (Cheret 2001). Inventions of other types of panel board are of much younger age. Until the middle of the 19th century, the abilities to mechanically crush wood and the industrial manufacturing of derived timber products were very limited. In 1861, wood fibre panels pressed from milled and grinded wood were patented for use as flooring and roofing tiles (Katlan 1994). First ideas of producing a wood-based panel similar to the present particle board were expressed in 1887 with a "Holzmasseplatte" ("wood-mass-board") to be made from sawdust and blood albumin (Hubbard 1887), and in a patent of the year 1889 that described gluing of wood chips length- and crosswise in parallel layers onto canvas. Until the 1940s, further investigations were conducted but it was only in 1941, when the world's first particle board factory was built by the Torfit-Werke G. A. Haseke in Bremen. Using strands from a close-by planer mill as the raw material combined with phenolic resins, the employers were able with a hydraulic platen press to produce up to 10 tons of one-layer particle boards of different widths (4 to 25 mm) and densities (0.8-1.1 g/cm³) per day. Unfortunately, the factory was bombed during the World War II and never rebuilt. At that time in Switzerland, the engineer F. Fahrni launched the idea from low grade wood material to make a three-layer particle board with an inner layer of coarse wood chips and outer layers of thin, laminar chips of special dimensions. In 1943, a pilot plant station was built in Klingnau, Switzerland and from 1946 onwards, 60 m³ of the NOVOPAN called three-layered particle board were manufactured per day. Considerable manpower was needed, since the particles were scattered by hand into a huge cast iron frame and, after pressing, the boards had manually to be removed. In 1949, the Holig GmbH in Sassenburg in Germany was the first worldwide that started automatic production of particle boards. The particles were scattered in continuous production lines, pre-pressed on steel plates and then conveyed to an automatically pulsing multi-platen press. Only ten years later, there were 62 particle board factories based in Germany working with large multi-platen presses that simultaneously could form up to 15 particle boards. Many of these early factories were owned by furniture manufacturers who intended to be self-sustainable by utilisation of their own wood waste materials. However, the market was quickly overhauled by a growing number of manufacturers like Triangel, Pfeleiderer and Moralt that specialised on particle boards, and also by price erosions (Lampert 1966, Deppe & Ernst 2000, Fischer 2002, 2003).

Particle boards today

Currently, particle boards comprise the largest part of all panel boards produced in Europe (Fig. 2). The total European production (Russia inclusive) in 2004 was about 37.6 million m³ and the German share about 9.3 million m³, an amount nearly as high as the total production of North America (Fig. 3). Total productions in Europe increased over the last 10 years to stagnate at the beginning of this millennium (Fig. 4). In the year 2001, with 32 factories, Germany was the European country with the highest production of particle boards (Mantau et al. 2002) whilst in 2002, there was a slight collapse in particle board production due to the continuous slump in the building and furniture industry (Fig. 3 and 4). The market saturated, causing an economically critical situation to the particle board manufacturers. Some factories in this situation were not able to resist the continuing market pressure and had to abandon their production (Mantau et al. 2002, Marutzky 2004). Sales in the particle board sector are however expected to recover, particularly with the eastern expansion of the European Union. This might be seen in the marginally better production rate in 2004 (Fig. 3 and 4) and following years (VHI 2007a). In addition, some regions in the world, especially the Far East (China, India, etc.), have a significant backlog demand for panel board products (Wemning 1997, Sun et al. 2005). It is also widely accepted that the consumers will buy more timber products in the future because of the constantly growing ecological awareness in the society. Investigations by Mantau et al. (2003) predict for the German particle board industry on the longer run an increase in capacity of about 10%. One fourth of the entire European production is assumed to be located in Ger-

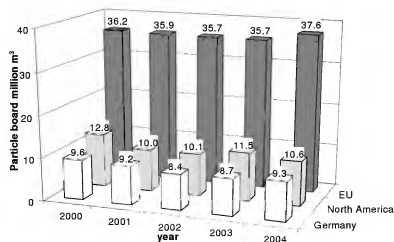


Fig. 3 Particle board production in Germany, Europe, and North America from 2000 to 2004 [according to EPF (2004), Alteheld (2007), and FAOSTAT (2007); values for North America have been obtained from subtracting values of OSB production (EUWID 2005) from FAOSTAT (2007) "particle board" data, that include both particle boards and OSB]

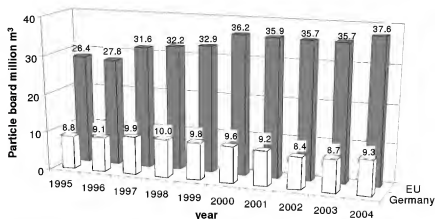


Fig. 4 Particle board production in Germany and Europe from 1995 to 2004 (after Mantau et al. 2003, Marutzky 2006; compare also the slightly different values of VHI 2007a)

many. With an annual exchange of 5.1 milliard € and 16,500 employees, Germany is the most important European panel board manufacturer (Kibat & Sauerwein 2006, VHI 2007b).

History of medium-density fibreboards (MDF)

Procedures for paper manufacture were the precursors on the way to develop today's fibreboards. In 1772, Henry Clay in Britain patented a thick, heat-resistant laminated paper panel called "papier mâché" that was made from wastes of paper and cellulose production. Till the late 19th century, these thick, non-flexible and firm paper grades found applications as carriage panels and building panels for houses, doors, and furniture (White et al. 1999). For papermaking in continuous sheets, at the turn of the 18th to 19th century, the Fourdrinier machine was invented with i. a wet section for loading pulp in slurry form and transferring it into a headbox from which the fibres are loaded on a moving wire for aligning them through the action of a vibrating Fourdrinier table, ii. a press section for removing water and pressing the formed mat through a system of rolls, iii. a dryer section build up by steam-heated rollers, and iv. a calender stuck for final smoothing the produced paper (Schubert 1992). With the invention of the Fourdrinier machine, also the mechanical premises for the manufacturing of fibreboards were created. However, industrial manufacturing of half-hard interfelted fibreboards started only about 100 years later in England, whilst insulating mats were produced in the US state Minnesota for building applications. In 1914, the first pilot plant station for the production of porous fibreboards from wood waste was installed in the USA. The first commercial hardboard plant followed in 1926 in Mississippi by the Mason Fiber Company which manufactured the patented Masonite-type fibreboards that are still produced today (FAO 1957, Lampert 1966, Katlan 1994). The success of these boards bases on the tremendous amounts of saw mill waste parti-

cles in the United States and the 1926 inventions by W.H. Mason for their defibration by high steam pressure. This steam explosion process known as Masonite-process (Boehm 1930) uses the thermoplastic behaviour of wood at temperatures of approximately 220°C. Wet particles are heated in a closeable tube to a steam pressure of 20 to 30 bar. Upon a sudden pressure release by opening the tube, the wood chips explode at atmospheric pressure into a muscoid material of mostly non-collapsed, rigid fibres. These fibres display a lignin-rich, highly reactive surface structure allowing the production of highly compressed and therefore hard high-density fibreboards (**hardboards**) without any adhesive or chemical additions (further reading in Chapters 16 and 18 of this book). In a slightly newer defibrator process patented by Asplund in 1931, humid wood chips are heated to 160 up to 180°C at a pressure of 8 to 11 bar in order to defibrate them by milling disks moving into opposite directions. Both, the steam explosive process and the defibrator process are applied today to produce high quality fibre material of technical and physical properties well suited for the fibreboard industry (Lampert 1966, Kotka & Ahmed 1997).

With the invention of suitable large-scale wood defibration processes, technological developments in the USA quickly resulted in the production of the medium-density fibreboards as a new generation of panel boards - a first MDF plant was built in 1965 by the Allied Chemical Company in Deposit, New York (Deppe & Ernst 1996). As the name indicates, this new type of panel board distinguishes from hardboard by density. MDF typically has a density of 600-900 kg/m³, hardboard a density of 900-1,200 kg/m³ and, for comparison, particle boards a density of 500-700 kg/m³ (DIN Deutsches Institut für Normung e.V. 1999). Compared to the earlier hardboards, MDF has a much better dimensional stability, a higher strength, a reduced weight, and two smooth sides. In addition, it can be produced in a greater range of thickness from 30 to 100 mm. Nowadays, by the calender, the Texpan, and the ContiRoll double belt pressing processes (Page 1984, Soine 1986), it is also possible to prepare thinner boards with gross densities in between those of light particle boards and the heavy hardboards. Thus, MDF finds much broader application than the hardboards, for example in the furniture industry and for interior fittings. Caused by the applied raw material and a less advanced process technology, the poor quality of American particle boards contributed further to the rapid propagation of MDF particularly in Europe (Deppe & Ernst 1996).

MDF today

MDF represents the second largest amount of panel boards produced in Europe (Fig. 1). Depending on the individual material, energy and capital costs in the different producer countries, MDF can be distinctly more expensive than particle board (compare e.g. import/export quantity and value data given by FAOSTAT 2007). Nevertheless, the constantly growing production rates document the breakthrough for MDF on the market (Fig. 5).

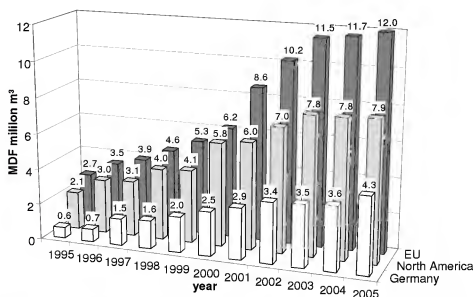


Fig. 5 MDF production in Germany, Europe, and North America from 1990 to 2005 (according to Thole & Marutzky 2006 and FAOSTAT 2007)

History of plywood and oriented strand boards (OSB)

Plywood is the oldest wood composite material known (Cheret 2001). Traces of laminated wood were found in tombs of Egyptian pharaohs. In China about 1000 years ago, in France and England in the 17th and 18th century, and in Russia prior to the 20th century, decorative hardwood has been glued together in a plywood manner for the manufacture of furniture and doors. In 1865, the first patent on plywood from softwood was given in the USA. Industrial fabrication of plywood started in 1905 in the City of Portland in Oregon in frame of a World's Fair with a product traded under the name 3-ply veneer work. First, such plywood was produced just for doors, cabinets and trunks but in 1920 the automobile industry became customers for plywood. A major breakthrough came in 1934 with the development of a moisture-resistant adhesive. Plywood became applicable to exterior use, and low cost "Dri-Bilt" homes were constructed with trademarked plywood subfloors, sheathings, ceilings, walls, and built-ins. In World War II, plywood was declared essential war material. It found multiple uses for boats, gliders, huts, and as parts of machines. Plywood production boomed after the war and it is till today the most produced panel board in the USA (Fig. 6) where the 100th anniversary of plywood industry was celebrated in 2005 (UNECE/FAO 2003, APA 2005).

OSB was first described in 1949 (Elmendorf 1949) but appeared on the market only in the late 1970s in order to compete with plywood as a structural material while using a low quality resource. It is a further development from the earlier produced **waferboards** (or **flakeboards**) and distinguished from the latter by size

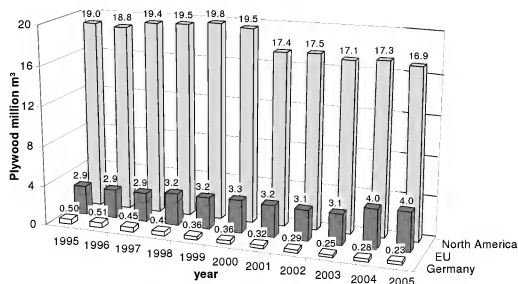


Fig. 6 Plywood production in Germany, Europe, and North America from 1990 to 2005 (data from FAOSTAT 2007)

and form of the strands. Strands in OSB are long, slender and oriented in right angles in contrast to the related waferboards that are built by large, plane and randomly arranged shavings. In consequence, OSB has much better strength than waferboards (Maloney 1993, Illston & Domone 2001). In the European Union, OSB is quite new (Fig. 7) with first standards defined in 1997. Accordingly, American OSB was first licensed in Europe in 1998 (FAS/USDA 1999).

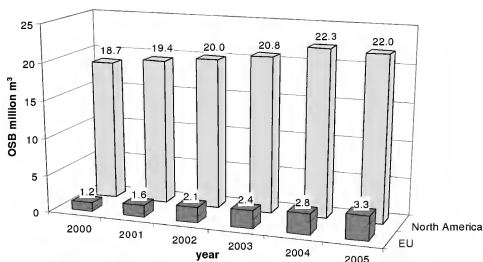


Fig. 7 OSB production in North America and Europe from 2000 to 2005 (data from EPF 2004, EUWID 2005, Krug & Tobisch 2006; see also VHI 2007a)

Plywood and OSB today

As shown in Fig. 6, production of plywood takes place mainly in North America with a slight reduction in quantities since the beginning of the millennium, whereas in Europe, respectively in Germany, production is low with no dramatic changes over the last ten years. In contrast, the production of the later invented OSB is steadily increasing both in North America and in Europe (Fig. 7). In North America, there is a high demand for OSB by the local construction trade (Meil et al. 2007). Particle boards are traditionally rarely employed in constructions on this continent, unlike in Europe where the building industry predominantly uses particle boards (EPF 2004, VHI 2007a).

Synthetic organic bonding agents

Predominantly **synthetic organic adhesives** are used as glues for the manufacturing of wood panels. About 90% of these bonding agents are **urea-formaldehyde resins** (Kharazipour 1996, Deppe & Ernst 2000).

Formaldehyde condensation resins

Formaldehyde condensation resins are mixtures of indefinite composition that are created by a chemical reaction of formaldehyde with urea (Fig. 8), melamine and phenol, and, in case of co-condensates, by combinations of these (Dunky 1998, 2004). In the production of such synthetic resins, formaldehyde is used either as aqueous formalin solution or in solid form as *para*-formaldehyde to form pre-condensates consisting of mainly oligomers. Formaldehyde condensation resins are direct or indirect thermosetting resins which in the cured condition predominantly contain methylene bridges from the reaction partner formaldehyde (compare Fig. 8). The complete curing to cross-linked polymers generally occurs under the influence of heat and/or catalysts, providing that these products still contain a sufficient number of reactive CH_2OH groups (methylol group; see Fig. 8). The final structure of the highly polymerised resins depends on the degree of condensation during the hardening process. During hardening, formaldehyde condensation resins can also form chemical bridges with the surfaces of wood fibres and particles (Dunky & Lederer 1982, Christjanson et al. 2006, Sümer et al. 2006, Stefke & Dunky 2006).

Aminoplastic resins

Aminoplastics are condensation polymerisation products of a nitrogen compound (most commonly urea or melamine) with an aldehyde (most commonly formaldehyde). By application of heat, aminoplast resins harden to insoluble and infusible macromolecules (Eisele & Wittmann 1990, Adam & Kamutzki 1993, Kharazipour 1996). These cheap and easily available resins have a fast curing time,

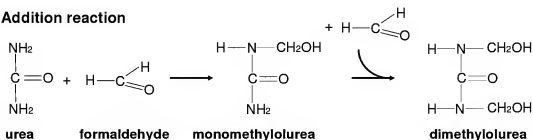
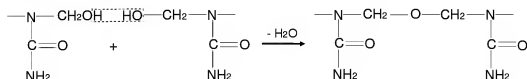
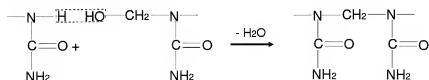
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Fig. 8 Condensation reactions of urea and formaldehyde to urea-formaldehyde resins (see Habenicht 1997, Christjanson et al. 2006)

provide a high bonding strength and are therefore the mostly used adhesives in the panel board industry (Eisele & Wittmann 1990, Weinkötz 2006).

Urea-formaldehyde resins (UF resins, UFRs) are the most important glue types of the aminoplast resins. Worldwide, the share of particle boards produced with acid-curing of UFR approaches 90% (Eisele & Wittmann 1990, Weinkötz 2006). To produce the actual adhesive, urea and formaldehyde are let in diluted solution to react with each other at an alkaline to neutral pH range (Christjanson et al. 2006). The reaction is stopped at the stage of the mono- and di-methylol-ureas (Fig. 8) in order to avoid formation of high-molecular methylol-ureas. Mono- and di-methylol-ureas represent the actual adhesive and are offered as solution with a solid content of approximately 66% or as powder (see Table 1 for adhesive properties). At acidic pH, dimethylol-groups continue to react, thereby forming condensation polymerisation products (Fig. 8). In practice, this is achieved through the addition of chemical catalysts, so called hardeners. Mostly, ammonium sulfate is used as the catalyst and less often ammonium peroxide sulfate or magnesium chloride (Xing et al. 2004, Steffe & Dunky 2006).

Table 1 Properties of common formaldehyde resins (Zeppenfeld 1991; Pfeleiderer Holzwerkstoffe GmbH & Co KG, Neumarkt, Germany, personal communication)

Attribute	Urea-formaldehyde	Melamine-formaldehyde	Phenol-formaldehyde
Appearance	Milky cloudy	Colourless/white	Red brown
Solid content	68 ± 1%	40-60%	45-60%
pH-value (20 °C)	7.5-9.5	7.5-8.1	12
Density (20 °C)	1.29-1.31 g/cm ³	1.29-1.31 g/cm ³	1.2 g/cm ³
Viscosity at filling in factory (20 °C)	300-500 mPa s	400-600 mPa s	400-600 mPa s
Hardening temperature	104 °C	Starting at 100 °C	130 °C
Required hardener addition	Yes	No	Only in the middle layer
Storage time	4-6.5 weeks	1-4 days (MUF up to 6 weeks)	3-12 weeks
Price (kg atro*) in 2006	0.40 €	1.30 €	1.20 €

* atro = "absolut trocken" = absolutely dry

In the manufacture of particle boards, liquid UFRs are diluted by water in order to decrease viscosity and facilitate spraying. Small amounts of hardener are added, as well as appropriate quantities of insecticides, wax emulsions and fire-retarding agents (such as ammonium phosphates) before spraying the glue mix onto the wood particles and pressing the boards. Pressing temperatures and maximum pressures for producing particle boards are in the range of 150 to 200 °C and 0.2 to 3.5 N/mm². The recommended moisture content of glue-impregnated furnish chips is 7 to 8% for the middle layer and 10 to 12% for the surface layers. For the middle layer, 6 to 8% of resin are used and 10 to 11% for the surface layer (Pizzi & Mittal 1994). Conditioning in cooling units (star coolers) after hot-pressing of the particle boards to cool at ambient temperature is necessary for postcuring in order to avoid any adhesive degradation and to improve particle board performance (Lu & Pizzi 1998, Deppe & Ernst 2000).

Formaldehyde is declared to be cancerogenic (Cogliano et al. 2005, IARC 2004, 2006, Schulte et al. 2006; see below for further details on health effects). Because of health concerns, legal restrictions exist for the usage of formaldehyde (ChemVerbotsV 2005). Earlier in panel board production, urea/formaldehyde (U/F) molar ratios of 1:1.4-1.5 were used and the resulting boards had formaldehyde levels of 50-70 mg HCHO/100 g board (Pizzi & Mittal 1994). Today's European standard for particle board allows 6.5 mg HCHO/100 g board and for MDF 7 mg HCHO/100g board, as measured by the perforator method (European standard EN 120 1992). Currently used UFRs mixtures keep with molar U/F ratios of 1:1.05 to 1:1.1 in the tolerated limits have however the disadvantages of a lower potential for dilution with water and of a longer curing time. Furthermore,

the produced panel boards have a lower water resistance because the resins are more vulnerable to hydrolysis (Pizzi & Mittal 1994, Dunky 1998, Kavvouras et al. 1998, Vargha 1998, Mansouri & Pizzi 2006, Park et al. 2006, Siimer et al. 2006).

Melamine-formaldehyde resins

Melamine-formaldehyde (MF) and **melamine-urea-formaldehyde (MUF)** resins are among the most commonly used adhesives for exterior and semi-exterior wood panels (plywood, particle board) and for the preparation and bonding of both low- and high-pressure paper laminates and overlays. Whilst their handling is very similar to UFRs, these resins provide a much higher resistance to water attack than UFRs. Pure MF adhesives (for properties of a typical MF resin see Table 1) are expensive, whereas MUF resins are cheaper by addition of urea (Pizzi & Mittal 1994, Dunky 1998). Applied mixed condensates usually consist of 45% MF resin and 55% UF resin. They are advantageous in particle boards in case of a short, temporary moisture exposition by restricting the swelling of the boards (Schwab et al. 1997, Deppe & Ernst 2000). However, under continued moisture exposition, also MUF-bonded particle boards swell strongly. Extra addition of phenol-formaldehyde (PF) resin to a certain degree can prevent this (Prestifilippo & Pizzi 1996, Prestifilippo et al. 1996, Zhou et al. 2001). Water and weather resistance can also be enhanced by addition of buffering additives that keeps the pH in the required acidic range where polycondensation occurs best. By an improved solubility, addition of melamine acetate salts can reduce the melamine content required for good water resistance. Addition of hexamine (Fig. 9B) as a hardener to MUF resins yields reactive unstable intermediates in the decomposition process of the compound. Hexamine under alkaline conditions easily reacts with the melamine that has very reactive nucleophilic sites (Fig. 9A). Also this can improve water and weather resistance (Kamoun et al. 2003, Zanetti & Pizzi 2003a,b, 2004). In industrial productions, star coolers are applied for postcuring of MUF-bonded panels (Zeppenfeld 1991, Deppe & Ernst 2000). Further to this, Zhao & Pizzi (2000) reported that after a period of cooling, a further step of thermal postcuring by hot-stacking of heated (90°C, 30 min) MUF-bonded panels can improve the internal bonding strength in particle boards without any further joint and hardened adhesive degradation in cases where adhesives of lower formaldehyde contents were applied.

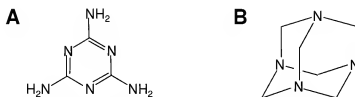


Fig. 9 Chemical structures of the nitrogen-rich melamine (A) and hexamine (B)

In MF resin production, the condensation reaction of melamine with formaldehyde is similar to the reaction of formaldehyde with urea (Fig. 8) but it is faster and is more complete. Formaldehyde first reacts with the amino groups of melamine (Fig. 9A) to form a series of methylol-compounds with two to maximum six methylol-groups (compare the reaction scheme presented in Fig. 8). In such a precondensation reaction, melamine with its amino groups accepts easily up to two formaldehyde molecules. Since melamine is not water-soluble, oligomerisation readily occurs through subsequent reactions between the left amino groups of different melamine molecules and methylol-groups coming from the formaldehydes. In practice, such reaction will be interrupted on time to obtain only short hydrophobic oligomers. MF precondensation to resins and the final curing (condensation) can occur under acidic, neutral and even slightly alkaline conditions. In the final curing, MF intermediates transform into insoluble and infusible MF compounds also through the reaction of amino and methylol groups, which are still available for reaction. The effective curing temperature range of MF resins starts at about 100°C and reaches the optimum at approximately 150°C. MF resins which cure under these settings evaporate a reduced quantity of formaldehyde as compared to UF resins under the same conditions (Pizzi 1983, Pizzi & Mittal 1994).

Phenolic formaldehyde resins

The third group of resins of significant importance for the panel board industry comprises **phenolic formaldehyde (PF) resins (phenol-formaldehyde resins)** which possess both a high dry and a high wet bonding strength, and a strong adhesion to wood (for detailed properties see Table 1). Phenol-formaldehyde resins have the broadest application range of all synthetic resins due to their high adaptability. Their main application field is the manufacture of humidity-resistant particle boards, but they are also used for the production of weather-proofed fibreboards, plywood, isolation boards, and for the generation of wafer- and oriented strand boards (Becker & Braun 1990).

Phenolic resins were the first true commercial synthetic polymers. Nevertheless, their structure is not completely clear since formaldehyde can react with the poly-functional phenols in the *ortho*- and the *para*-positions to the hydroxyl group. In consequence, the condensation products exist as numerous positional isomers for any chain length (Pizzi & Mittal 1994, Lei et al. 2006, Fig. 10). Upon condensation, PF resins will not further deform. Therefore, these types of binders are classified as **duroplasts** (Becker & Braun 1990).

PF resins are produced in a discontinuous process in the presence of sodium hydroxide (NaOH) at a molar ratio of phenol to formaldehyde of about 1:2.5 (Becker & Braun 1990). NaOH has the effect of decreasing the activation energy in resin curing (Grenier-Loustalot et al. 1996, Pizzi et al. 1997, Tonge et al. 2001,

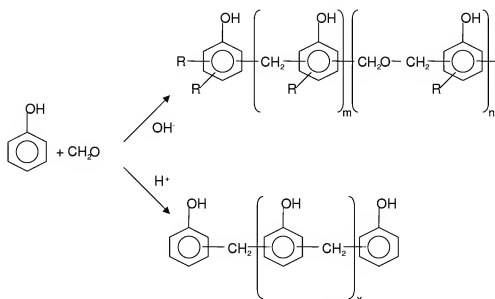


Fig. 10 Polycondensation reactions of phenol-formaldehyde resins – resol is formed when OH^- is used as catalysator (top), novolak with H^+ as catalysator (bottom)

Park et al. 2002). However, because of the high hygroscopic character of this catalyst, the equilibrium moisture content of wood raises with the consequence that, compared to other adhesives, press times have to be increased. The final curing of PF resins occurs under the elimination of water between the reactive hydroxymethyl groups and the detachable hydrogens at the phenolic core. New methylene bridges are formed. Linear structures arise and, with growing condensation level, crosslinking bridges are created between phenolic groups of different linear structures (Roffael & Schneider 1981, Kharazipour 1996; compare Fig. 10).

PF resin starts the final curing at approximately 130°C . The speed of curing is linked to temperature. With increasing temperature, the curing speed becomes progressively shorter (Umemura et al. 1995, Wang et al. 1995, Geimer & Christiansen 1996). After hot-pressing, PF resin-bonded panel boards require further hardening by hot-staking to achieve full curing. Unlike in application of UF and MUF resins, usage of star coolers for postcuring in industrial manufacturing is not mandatory for PF resins. By the ongoing hardening of incompletely cured PF resins during hot-staking, energy costs can be economised by applying shorter pressing times than required for the full condensation reaction (Zeppenfeld 1991, Deppe & Ernst 2000). As a further advantage, elimination of formaldehyde from PF resin-bonded panel boards is approximately 10% lower than that of UF resin-bonded panel boards (Cherubin 1978, Wang et al. 2003). In spite of this, the pressing conditions as applied of course very much influence the final emissions from the boards (Petinarakis & Kavvouras 2006).

Not completely cured bonding layers and those, which may release free phenols, are not allowed for the use of furniture and indoors where foods are stored (Zeppenfeld 1991). The toxic and chemically aggressive phenols can have negative effects on health (Bruce et al. 1987, Horch et al. 1994, Isaksson et al. 1999) but fully cured PF resins are hygienically harmless. In contrast to UF resins with their easily to split C-N bonds (Fig. 8), hardened PF resins linked by stable C-C bonding (see Fig. 10) cannot be damaged hydrolytically by moisture or heat (Dunky 2004). The type of resin used in board production influences the water retention values of particles and fibres (Roffael et al. 2003). The equilibrium moisture content of PF resin-bonded particle boards is up to 10% higher than that of UF resin-bonded particle boards (Roffael & Schneider 1978).

Isocyanates (polyurethanes)

Organic isocyanates, usually **polymeric methylene diphenyl diisocyanate (PMDI)**, see Fig. 11), have been used as adhesives since the beginning of the 1970s, mainly in particle board fabrication (Larimer 2006). Isocyanates, characterised by the $-N=C=O$ group (see Fig. 11), are not adhesives like the above mentioned thermosetting bonding agents. They do not have any cold tackiness and cure with water only to a foamy compound of lower strength. Therefore, special emulsifiers have to be added as solvents to create an utilisable paste for panel boards (Boeglin et al. 1995).

PMDI (Fig. 11) is produced by phosgenation of aniline formaldehyde condensates. Above 25°C, PMDI reacts with water in form of gelification. At higher temperatures and pressures, firm varnish coatings will generate in which 20-50% of

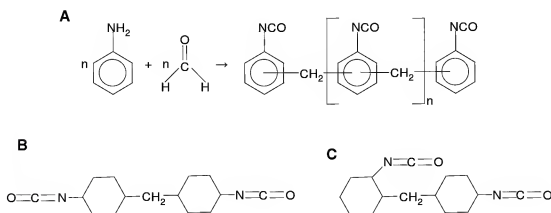


Fig. 11 A. Condensation of PMDI (polymeric methylene diphenyl diisocyanate) from aniline and formaldehyde. B. 4,4'-MDI (4,4'-diphenylmethane diisocyanate) and C. 2,4'-MDI (4,4'-diphenylmethane diisocyanate) as two major forms of diisocyanates present in PMDI mixtures used as binders for wood (Johns 1982)

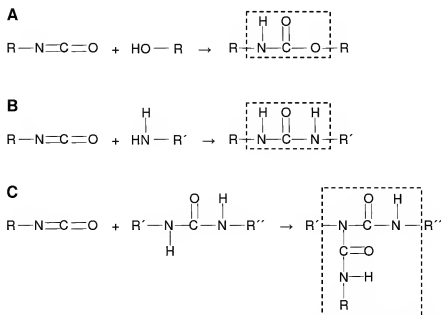


Fig. 12 Formation of polyurethane, polyurea, and biuret-type structures. A. An urethane structure (also known as carbamate structure; encircled) forms when a difunctional isocyanate reacts with a difunctional alcohol. B. Reactions between isocyanates and amines yield polyurea (urea structure encircled). C. Reactions of between isocyanates and urea compounds gives rise to compounds with a biuret group (encircled). After Johns (1982) and Becker & Braun (1990)

the PMDI are transformed into polyurethane, whereas the rest is polyurea (Fig. 12). PMDI interacts well with wood and other lignocellulose-containing materials and creates powerful adhesive joints during hot curing (Dunky & Niemz 2002). The level of crosslinking between PMDI and the wooden material rises with increasing temperature (Boeglin et al. 1995). PMDI likely reacts with the water enclosed in the materials under release of CO_2 to polymeric ureas (polyurea) and possibly further to biuret structures and, in addition, it reacts via addition reactions with hydroxyl groups provided on the surface of lignocellulose to polyurethanes (Johns 1982, Becker & Braun 1990; Fig. 12). Investigations have shown that the isocyanates undergo in wood chemical reactions with lignin, cellulose, and other cell wall compounds (Johns 1982, Boeglin et al. 1995, Bao et al. 2003). Both urea and biuret structures can be clearly recognised in the PMDI-bonded wood composites (Wendler & Frazier 1995, 1996, Ni & Frazier 1998, Bao et al. 2003) and there is also experimental evidence for the formation of urethane (Das et al. 1997, Zhou & Frazier 2001). The covalent chemical bonds formed between PMDI and wood surfaces within panel boards are much stronger, more durable and more moisture-resistant than those mediated by UF, MUF, and phenol resins (Zeppenfeld 1991, Deppe & Ernst 2000). Another positive effect of usage of

PMDI is that formaldehyde emissions from pressed boards are negligible (Wang et al. 2007). Negative for application of PMDI in panel board production is its high price (in 2006 about 2.0 €/kg *atro, atro* = “absolut trocken” = absolutely dry; Larimer 2006). Furthermore, during application of isocyanates in practice significant protection measures are necessary (Zeppenfeld 1991, Petsonk et al. 2000) in order to avoid possible health risks and occupational diseases (among others, reactions with amino- and hydroxide groups within the body upon inhalation, destruction of cell membranes, respiratory diseases, skin eczemas, conversion into carcinogenic amines; for reports on health effects see e.g. Baur et al. 1994, Bolognesi et al. 2001, Goossens et al. 2002, Marczynski et al. 2003, Latza & Baur 2005). A technical problem in the application of the highly reactive isocyanates is the risk of them sticking to metallic parts of the hot press (Dillingham & Moriarty 2003), why releasing agents or specially coated conveyor belts must be used (Zeppenfeld 1991).

New developments

Various types of panel boards are well established on the market. However, further developments are possible regarding new types and combinations of fibre and particle materials, glues, combinations of fibres and particles with non-plant material, arrangements of material layers and process conditions – all of which will influence the properties of the resulting products. Wood composites need to meet certain standards in technical properties such as dimension stability, bending strength, and thickness swelling. These are defined by European standards (DIN Deutsches Institut für Normung e.V. 1999; Tables 2 to 6). Technical equipment required for testing such properties is shown in Fig. 13.

The various types of applications of wood composites may ask for choices of different specifications of boards such as higher or lower density and weight, for different assortments in thickness, and for specific adaptations of boards to dry or wet areas. Regional differences e.g. in material availability and climate conditions require adaptations to local conditions. Last but not least, concerns for health and environment request safe products.

Volatile (formaldehyde) emissions of wood-based board materials must be under defined low values (Dunky 2006; Tables 2-6). The fast perforator method explained in Fig. 14 as one way to determine formaldehyde emissions (European standard EN 120 1993) is convenient for routine application in industry and research. In the time-consuming chamber methods, formaldehyde evaporated from a sample of defined size at defined temperatures and humidity is mixed with air in a system of fixed volume (the chamber) and air flow. Specific volumes of air are sampled twice a day for formaldehyde concentration determination (European standard EN 717-1 2004, Japanese standard JIS A 1901 2003). The chamber methods are reference methods for approval of new types of boards and in court.

Table 2 Tests for particle boards defined by European standards: X = tests required

Test	Particle board type (EN 312-2 to 312-7 2003)*					
	EN 312-2	EN 312-3	EN 312-4	EN 312-5	EN 312-6	EN 312-7
Thickness swelling (EN 317 2003)			X	X	X	X
Internal bond strength (EN 319 1993)	X	X	X	X	X	X
Bending strength (EN 310 1993)	X	X	X	X	X	X
Modulus of elasticity (EN 310 1993)		X	X	X	X	X
Surface soundness (EN 311 2002)		X				
Formaldehyde emission (EN 120 1992 or EN 717-3 1996)	X	X	X	X	X	X
Option 1:						
Alternatives Thickness swelling in cyclic test (EN 317 2003 + EN 321 2002)		X		X		X
Internal bond strength in cyclic test (EN 319 1993 + EN 312 2003)						
Option 2:						
Internal bond strength after boil test (EN 319 1993 + EN 1087-1 1995)		X		X		X

* Boards for general use (EN 312-2 2003), interior fitments including furniture (EN 312-3 2003), subjected to stress (EN 312-4 2003) and high stress (EN 312-6 2003) in applications in dry areas; boards subjected to stress (EN 312-5 2003) and high stress (EN 312-7 2003) in applications in wet areas

Table 3 Tests for MDF defined by European standards: X = tests required

Test	MDF type (EN 622-5 2006)*			
	MDF	MDF.H	MDF.LA	MDF.HLS
Thickness swelling (EN 317 2003)	X	X	X	X
Internal bond strength (EN 319 1993)	X	X	X	X
Bending strength (EN 310 1993)	X	X	X	X
Modulus of elasticity (EN 310 1993)				
Formaldehyde emission (EN 120 1992 or EN 717-3 1996)	X	X	X	X
Option 1:				
Alternatives Thickness swelling in cyclic test (EN 317 2003 + EN 321 2002)		X		X
Internal bond strength in cyclic test (EN 319 1993 + EN 312 2003)				
Option 2:				
Internal bond strength after boil test (EN 319 1993 + EN 1087-1 1995)		X		X

* MDF and MDF.H: boards for general use in applications in dry and wet areas, respectively; MDF.LA and MDF.HLS: boards subjected to high stress in applications in dry and wet areas, respectively (EN 622-5 2006)

Table 5 Tests for plywood defined by European standards: X = tests required

Test	Plywood types*		
	EN 636-1	EN 636-2	EN 636-3
Dimension tolerance (EN 324-1 1993, EN 324-2 1993)	X	X	X
Bending strength (EN 310 1993)	X	X	X
Density (EN 323 1993)	X	X	X
Quality of bonding (EN 314-1 2005)	X	X	X
Formaldehyde emission (EN 717-2 1995)	X	X	X
Size alteration, appearance of the surface, suitability for finishing (EN 318 2002)	X	X	X
Traction-, shear- and compression properties (EN 789 2005)	X	X	X
Resistance to axial withdrawal of screws (EN 320 1993)	X	X	X
Capacity properties (floors, walls and roofs) (EN 1195 1998, EN 594 1996, EN 596 1996)	X	X	X

* Plywood types (EN 636 2003): Boards for applications in dry areas (EN 636-1 2003), in wet areas (EN 636-2 2003), and for outdoor applications (EN 636-3 2003)

Table 6 Tests for OSB defined by European standards: X = tests required

Test	OSB types (EN 300 2006)*			
	OSB/1	OSB/2	OSB/3	OSB/4
Thickness swelling (EN 317 2003)	X	X	X	X
Internal bond strength (EN 319 1993)	X	X	X	X
Bending strength (EN 310 1993)	X	X	X	X
Modulus of elasticity (EN 310 1993)	X	X	X	X
Formaldehyde emission (EN 120 1992 or EN 717-3 1996)	X	X	X	X
Option 1:				
Internal bond strength after boil test (EN 319 1993 + EN 1087-1 1995)			X	X
Bending strength in cyclic test (EN 310 1993 + EN 321 2002)				
Option 2:				
Internal bond strength in cyclic test (EN 319 1993 + EN 321 2002)			X	X

* Boards for interior fitments including furniture in applications in dry (OSB/1) and wet (OSB/4) areas, respectively; boards subjected to stress in applications in dry (OSB/2) and wet areas (OSB/3), respectively (EN 300 2006)



Fig. 13 Universal test machine (Zwick/Roell ZO10, 10 kN test load, Zwick/Roell, Ulm, Germany) in the "Biotechnikum" (pilot plant station) at the Institute of Forest Botany in Göttingen used for determining mechanical-technological properties of wood-based panels and of solid wood. A. Test equipment for determining surface strength of wood-based panels. B. Test equipment to determine internal bond strength of wood composites and of solid wood. C. Test equipment for determining bending strength and modulus of elasticity for composites and solid wood. Forces are applied by the test machine to samples of defined sizes until these break. Corresponding values are measured and transferred to a computer where they are analysed by a special software

Further derived alternatives for fast detection of formaldehyde emissions are the gas analysis method and the flask method defined in European standards EN 717-2 (1995) and EN 713-3 (1996) and the desiccator method defined in the Japanese standard JIS A 1460 (2001). When applying these methods it has however to be noted that results from different standard methods are not necessarily directly comparable (Roffael 1993, Dunky 2006, Que & Furano 2007, Que et al. 2007, Risholm-Sundman et al. 2007).

Depending on the respective applications, boards need to resist various biological threats, mediated by certain insects and different types of microbes (see Chapter 13 of this book). For implementation of new types of panel boards, their



Fig. 14 Overview and detailed view of perforators in the chemical laboratory of the "Bio-technikum" at the Institute of Forest Botany in Göttingen used to measure formaldehyde emission of wood based panels according to European standard EN 120 (1993). Samples of fixed sizes (25 mm x 25 mm) are given into round bottom flasks, filled up with 600 ml of the solvent toluene, and boiled up in order to collect the formaldehyde in 1000 ml distilled water filled into the perforator elements attached to the round bottom flasks. After boiling for 2 h, the distillates are harvested from the perforator elements, mixed in appropriate volumes with acetyl-acetone and ammonium acetate, and incubated for 1 h at room temperature in the dark. The formaldehyde in the distillates will react with acetyl-acetone and ammonium acetate to form the yellow, strongly fluorescing 3,5-diacetyl-1,4-dihydropyridine derivative. The concentrations of the 3,5-diacetyl-1,4-dihydropyridine derivatives are measured in a photometer at a wave length of 412 nm and used to calculate the formaldehyde concentration in the original samples

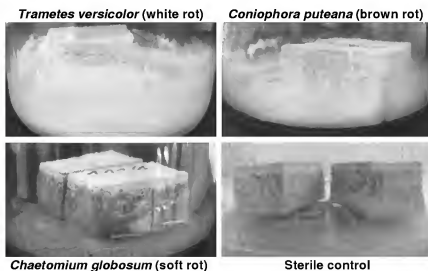


Fig. 15 Fungal decay test based on European standard EN 113 (1996) of particle boards made by wood chips of *Abies grandis* in the outer layer and of *Fagus sylvatica* in the inner layer (sandwich particle boards; see also Fig. 21)

behaviour with white-rot, brown-rot and soft-rot fungi have to be tested (Fig. 15), also following standardised tests (European standard EN 113 1996).

New applications for boards demanding altered specifications, regional feed-stock availability, local environmental conditions, safety concerns, and also political issues are apparent reasons for active research in the field of wood composites. For inventing and implementing new panel boards, extensive research and development work has to be done. To this end, pilot plants (see Fig. 16 to 20) are needed to test new material - adhesive reaction mixtures and reaction conditions at an appropriate scale, large enough to evaluate the technical properties of novel panel boards and judge their application potential before running the very costly production trials at an industrial scale.

New types of panels

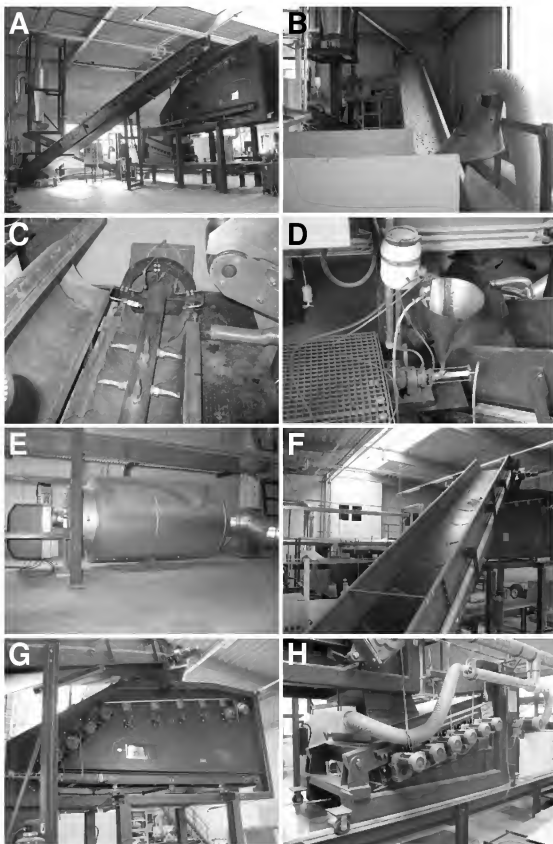
The most common raw materials for industrial production of fibre- and particle-boards are softwoods such as pine and spruce. Small amounts of hardwood, e.g. beech and poplar, might be added. However, the fraction of beech must not exceed 20% of the total wood. Beech (and also some other hardwoods such as oak) presents a risk of allergies and cancer to the workers in the wood industry due to an occupational hazard through dust generated when finishing plates by sanding (Nelson et al. 1993, Zhou et al. 1995, Wolf et al. 1998, Palus et al. 1999, Naaralla et al. 2003, Määttä et al. 2005). However, such problem is overcome when problematical wood is banned to the inner layer of multi-layered plates (Fig. 15 and 21).

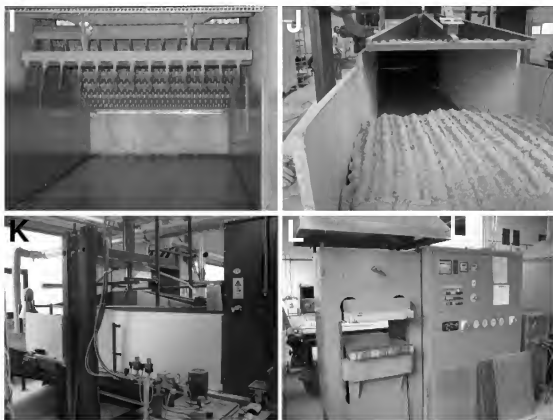
Many other types of wood and even clonal variation within a given tree species have still to be investigated for their suitability in panel board production (Shi et al. 2005). Behaviour with adhesives for example can differ fundamentally between different wood species (Kamke & Lee 2007). In times of shortness of wood, fast growing species such as *Abies grandis* (grand fir) are tested in their qualities for composite wood production to generate innovative panel boards for construction purposes as well as to establish new light-weighted material for insulation (Fig. 21). New techniques of wood fragmentation give additional options for designing novel types of panel boards (Joscak et al. 2006), as combinations of differentially shredded and shaved material types (Kawasaki & Kawai 2006). Naturally fermented wood from the forests (i.e. wood from partially decayed stems and branches) presents another, so far not exploited resource (see Chapter 18 of this book). Recycling of used board material in production of new panel boards is another important issue for ecosystem quality (Rivela et al. 2006; see Chapter 20 of this book). Combinations with other type of material - plastics, cement, ceramic, gypsum, glass fibres, etc. (see below) - is also possible (Karam & Gibson 1994, Fuwape & Oyagade 1993, Al Rim et al. 1999, Clemons 2002, Okamoto 2003, Hunt & Vick 2004, Jiang & Kamdem 2004, Jorge et al. 2004, Timar et al. 2004, Bastian et al. 2005, Kent 2005, Chen et al. 2006, Deng et al. 2006, Najafi et al. 2006, Qi et al.



Fig. 16 MDF pilot plant in the "Biotechnikum" at the Institute of Forest Botany in Göttingen. This first fully-automatic pilot plant in Germany was developed and constructed in cooperation with the company BINOS Technologies GmbH & Co. KG (Springe, Germany). The construction and technical equipment matches industrial plants and allows fully-automatic bulking, gluing, drying, and spreading of fibres (see Fig. 17 for further details). The pilot plant can produce fibreboards up to 20 mm in thickness by dry-, semi-dry, and wet-processes. It can be operated with all kinds of fibre material (wood, hemp, flax, straw, etc.) and conventional resins as well as natural binder systems. The pilot plant is used mainly for research on alternative raw materials and new bonding agents and for development of new generations of fibreboards

Fig. 17 The process of MDF production on pilot scale. A. Full view of the pilot plant in the "Biotechnikum" at the Institute of Forest Botany in Göttingen (shown from the other side than in Fig. 16). B. Conveyor belt transporting fibres into the blender for mixing with glues. C. View into the opened blender used for dry-gluing – note the three injectors (see Fig. 18 for a larger view) used to spray bonding agents onto the fibres. D. Pump to deliver bonding agents from the funnel to the three injectors within the blender. E. Gas-heated convection dryer for drying the fibres after blending. F. Large conveyor belt for the transport of the dried fibre material into the bunker (dosing bin). G. View of the bunker filled with fibre material that with the help of delivery rollers is further transferred into the mat-forming station. H. Outer view of the mat-forming station showing the





engines operating the spike rollers required for spreading the fibre material for mat formation. I. Detailed view of the inner spreading machine consisting of seven in opposite directions acting spike rollers that loosen, distribute and comb the material into a non-woven mat. J. Conveyor belt to transport the non-woven mat to the cold-press for pre-pressing. K. Cold-press acting with constant pressure of 50 bar. L. Hot-press acting under temperatures up to 240 °C and maximum pressure of 250 bar for final pressing

2006, Shimazu et al. 2006, Smith & Wolcott 2006, Zhang et al. 2006, Bilba et al. 2007, and others), further broadening the range of applications for wood of poor quality: generally as material in construction and specifically also as (light-weight) material providing thermal and acoustic insulation (Sekino & Yamauchi 2007) and even as material able to act as fire-shields (Harada et al. 2006).

New types of fibres

In general, fibres can be classified into three categories: **wood**, **non-wood**, and **non-plant**. The term “non-wood” was coined to distinguish other types of plant fibres from the two main sources of wood fibres, hardwoods and softwoods. Non-wood or agro-based fibres are derived from selected tissues of various mono- or dicotyledonous plants (Parham & Kausfinen 1974). Non-wood or agro-based fibres are a potential source of feedstock for the construction material in-

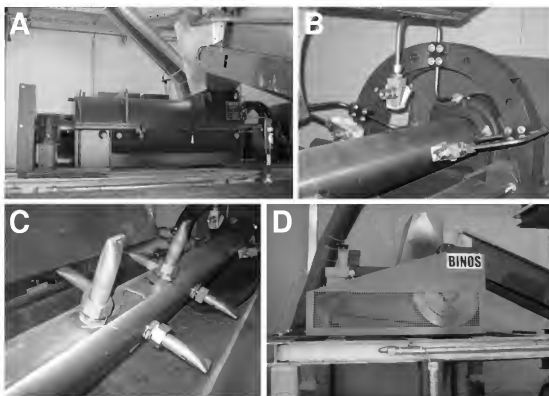


Fig. 18 The MDF pilot plant in the "Biotechnikum" at the Institute of Forest Botany in Göttingen is equipped with a blender for spraying the fibres with glue. A. Closed view of the blender. B. and C. Views on the interior of the blender showing the three injectors for the binders and the spindle with paddles for mixing and transporting the fibre material. D. Motor driving the spindle

dustries that will allow them to move away from the current reliance on wood materials (Durst et al. 2004). Agricultural residues are for many reasons an excellent alternative to using wood materials. Apart from their abundance and ability of renewal, using agricultural residues in industrial purposes is a much more environmentally friendly practice than residue disposal methods such as burning or land filling.

Globally, many lignocellulosic material options exist for the production of composite products, both in industrialised as well as in developing countries. Tested in production of particle- and fibreboards were for example wheat straw (Han et al. 2001, Mo et al. 2003, Boquillon et al. 2004, Schirp et al. 2006a,b), paddy straw (Sampathrajan et al. 1992, Hiziroglu et al. 2007), reed (Han et al. 2001, Habibi 2006), *Miscanthus* fibres (Velasquez et al. 2002, Salvadó et al. 2003), *Agropyron* straw (Zheng et al. 2007), bamboo (Zhang et al. 1996, Chen et al. 1998, Ma et al. 1998, Bai et al. 1999, Nugroho & Ando 2000, Sumardi et al. 2006, Hiziroglu et al. 2007), hemp fibres (Boquillon 2006, own research), flax (Papadopoulos & Hague

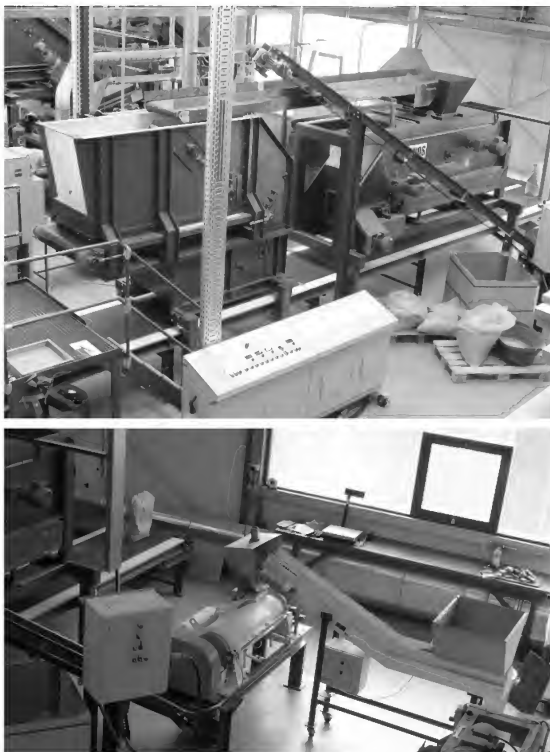


Fig. 19 Pilot plant for particle board production in the "Biotechnikum" at the Institute of Forest Botany in Göttingen. Top: two bunkers in line allow the production of three-layered boards. Bottom: particles are transported via a conveyer belt into the blender (further details in Fig. 20)

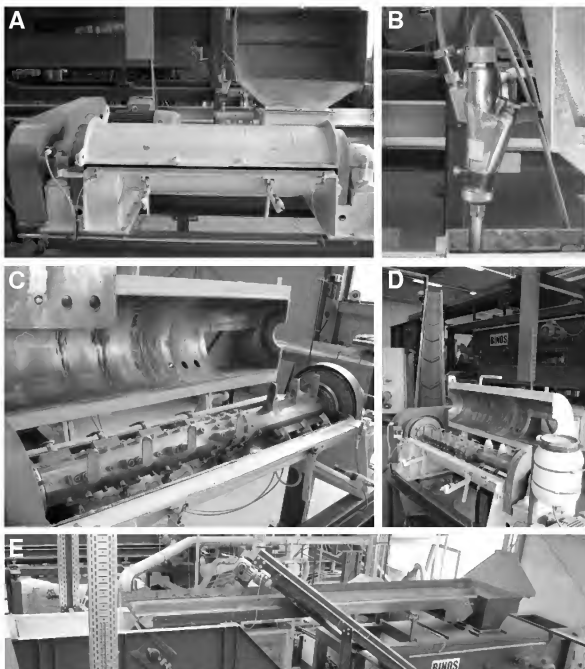
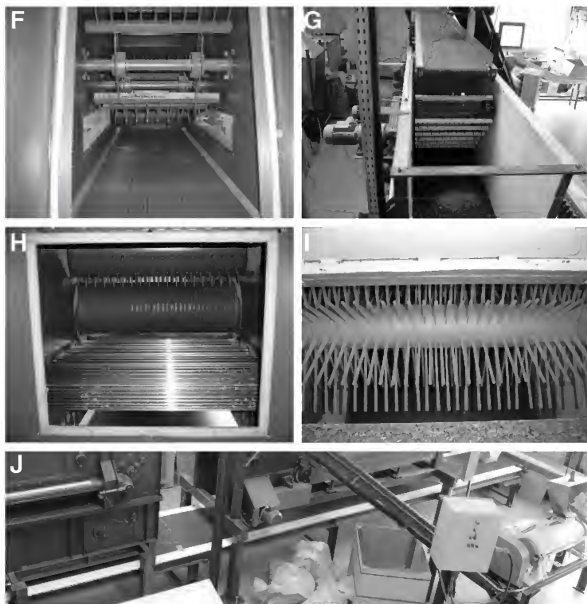


Fig. 20 The process of particle board production on pilot scale in the "Biotechnikum" at the Institute of Forest Botany in Göttingen (compare Fig. 19). A. View of the closed blender with a funnel for filling up particles and an injector for the binder. B. Large view of the injector. C. View into the opened blender: the large opening in the lid is the inlet of the particles and the tip of injector is seen in the front most round hole in the lid (see also the inset in the upper left corner). Note the different paddles on the spindle. Strong paddles underneath the large inlet help to press the incoming particles together and push them underneath the injector to be doused with glue. The buckled paddles mix the binder into the particle mass and transport the material to the outlet in the bottom end of the blender. D. The particles fall then onto a conveyor belt that transport them onto



another conveyor belt above the two bunkers of the pilot plant. E. This second conveyor belt can move into two directions for transferring either finer particle material for the outer layers of particle boards into the bunker 1 (in the photo right) or coarser particle material for the inner layer into the bunker 2 (in the photo left). F. View into bunker 1 with spike rollers for transporting, dosing, and spreading material for outer layers. G. View into bunker 2 with different types of spike rollers for transporting, dosing and spreading material for the inner layer. H. Scatterhead in the bunker 1 for discharging fine particles for the outer layers into casting trays that first move on a conveyor belt underneath bunker 1 in direction towards bunker 2. The most interior thrown particles are of smallest size giving rise to the fine and dense surface structure of the finalised boards whilst particles thrown up more closer on the way to bunker 2 are of increasing size for a changeover to the inner layer. I. Scatterhead in bunker 2 for discharging larger particles for the inner layer of particle boards into casting trays moving underneath

the bunker 2. J. Conveyer belt transporting casting trays with the growing first outer layer first from bunker 1 to bunker 2 to fill them there with the material for the inner layer and then transporting them back from bunker 2 to the scatterhead of bunker 1 in order to form the second outer layer by sieving material with decreasing grain size into the trays and to give also a fine dense structure to the second surface of the panel boards. Once material filling is finished, the casting trays are transported to the press shown in Fig. 17L for hot-pressing the material

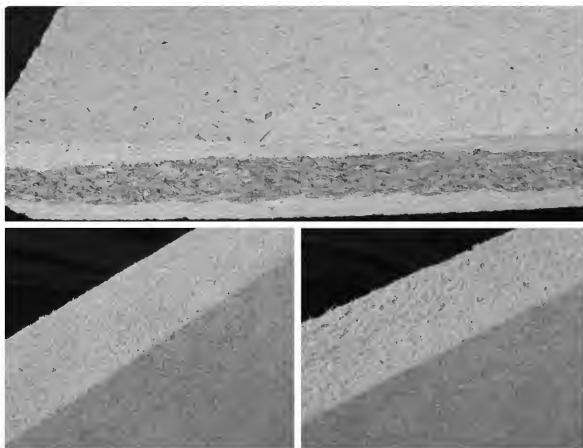


Fig. 21 Sandwich particle board with an outer layer made from grand fir wood chips and an inner layer of beech wood chips (top) and new types of insulating material made from grand fir fibres (bottom right), respectively beech wood fibres (bottom left). Note the different surface structure of the insulation material from the different wood species

2003), jute fibres (Deng & Furuno 2002), kenaf core (Xu et al. 2005, 2006), cotton stalks (Guler & Ozen 2004), banana fibres (Bilba et al. 2007), sun-flower stalks (Khristova et al. 1998, Guler et al. 2006) and peels (Boehme 1993), corn stover (Ren et al. 2006) and maize cobs (Sampathrajan et al. 1992), rice husks (Panthapulakkal et al. 2006), peanut shells (Batalla et al. 2005), almond husks (Crespo et al. 2007), durian peels (Khedari et al. 2004), coconut stem chips (Papadopoulos

et al. 2002), pith (Sampathrajan et al. 1992), and coir (Khedari et al. 2004), palm fronds (Suzuki et al. 1998, Laemsak & Okuma 2000, Hosseinkhani & Kharazipour unpublished), sugarcane bagasse (Widyorini et al. 2005), sisal (Agopyan et al. 2005, Tonoli et al. 2007), rattan (Olorunnisola & Adefisan 2002), and also newsprint (Nada & Hassan 1999) and other types of waste papers (Ellis et al. 1993, Massijaya & Okuma 1996, Grigoriou 2003, Le Fur et al. 2004, Hwang et al. 2006). However, these various types of fibres have tremendous variations in chemical and physical properties as compared to wood fibres. Accordingly, production conditions with such special materials might differ from those of production with conventional wood chips and fibres. In addition, produced boards have quite distinct characteristics and thus potential applications (for details see cited references).

Non-plant fibres include **natural asbestos fibres** and **man-made glass fibres**, rock wool, and other mineral fibres. Asbestos fibres served for over 100 years as insulation and fire prevention material but have been banned by now in many although not all countries by the high incidences of chronic bronchitis, lung fibrosis, and lung cancers provoked by the material (Osinubi et al. 2000, Greenberg 2004, Joshi et al. 2006). Asbestos fibres have been replaced in insulation and fire prevention material in some cases by wood fibres (Negro et al. 2005) and other low cost natural fibres (Savastano et al. 2004) but more often by **man-made vitreous fibres (MMVF)** (Christensen et al. 1993) being considered as relatively innocuous to human health (Hesterberg & Hart 2001). The synthetic types of inorganic fibre materials might be used solely or in combination with wood particles, fibres, and dust or with other plant material. In wood-polymer composites (**WPC, wood-plastic composites**; i.e. composites made of wood and polymers such as polyethylene, polyvinyls, polypropylene, etc; Schneider 1994, Ellis 2000, Faruk et al. 2007), addition of MMVF can help to reinforce the material, enhance their mechanical and physical properties and their durability. Glass fibres and mica fibres are e.g. sometimes added to PVC [poly(vinyl chloride)]/wood flour composites to confer better impact strength, flexural strength, and flexural toughness (Jiang et al. 2003, 2007, Jiang & Kamdem 2004). Mixtures of wood chips and basalt fibres in polymer composites are tested to increase flame retardancy and mechanical properties of material for the use as heat and sound insulating panels in automobile industry (Matko et al. 2006). Since WPCs are relatively new inventions (Schneider 1994, Wolcott & Englund 1999, Clemons 2002), there is a large potential of development in this field.

New type of binders

Life cycle assessments reveal that synthetic binders differ in economical and ecological preferences. Calculating for example SO_x emissions and energy costs, UF resins perform better than PF and API resins (aqueous polymer isocyanates; in German EMDI for "emulgiertes PMDI", Zeppenfeld & Grunwald 2005) and these in turn better than MUF resins. API resins in contrast show highest CO₂

and NO_x emissions (Sawada et al. 2006). Eco-efficient bio-based adhesives from renewable, natural, non-oil-derived raw materials are in focus to substitute synthetic resins. Raw materials for such bio-based adhesives are e.g. tannins, lignins and liquefied wood, carbohydrates, unsaturated oils, and proteins (Pizzi 2006). Chapter 16 of this book describes the current progress in the formulation of various types of bio-based glues.

Previously, organofunctional silanes, synthetic unbranched or branched organic compounds that possess a silicon-carbon (C-Si) bond (Jones et al. 2000), have been described in the literature as coupling agent in glass fibre/polyethylene-reinforced composites (Bikiaris et al. 2001). Organofunctional silanes have also been reported to act as wood preservatives (Hill et al. 2004). Work on organofunctional silanes in our institute lead to new binders for wood composites. These new binders on basis of organofunctional silanes give panel boards of excellent technical quality (Edelmann et al. 2007a,b, Jenkner et al. 2007a-d, Kloeser 2007, Kloeser & Kharazipour 2007). Formaldehyde emissions from purely silane-bonded boards are lower than the natural formaldehyde emissions from wood suggesting that the silanes have scavenger function to formaldehyde (Kloeser 2007).

The cheap UF resins are nontoxic in their cured state but during handling in panel board production and later in use by decomposition of the hardened glue, formaldehyde might escape from boards (Dunky 1998, see above). Formaldehyde is an issue of environmental concern by its adverse effects on health and has recently been upgraded by the World Health Organization (WHO) to hazard class 1, "carcinogenic" (Cogliano et al. 2005, IARC 2004, 2006, Schulte et al. 2006; see above). Critical health effects of formaldehyde exposure include sensory irritation and inflammation of the mucous membranes of the eyes, nose, and mouth, allergic reactions, and the potential to form protein-protein and DNA-protein cross-links in cells of the body and to induce tumors particularly in the upper respiratory tract (see for example the studies by Basketter et al. 2001, Vasilescu et al. 2004, Arts et al. 2006, Casset et al. 2006, McGregor et al. 2006, Metz et al. 2006, Speit & Schmid 2006). In addition, high temperatures and high relative humidity can result in odour problems in rooms containing panel boards manufactured with UF resins (Marutzky 1989, Kulle 1993). European standards EN 622-1 (2003), EN 300 (2006), and EN 636 (2003) provide for panel boards in use (particle boards, MDF, OSB, respectively) the definitions of emission class E1: i.e., emissions must not exceed 8.0 mg/100 g absolutely dry particle board as measured by the perforator method. In Japan, the class F** limit of formaldehyde emission of panel boards in interior use is ≤ 1.5 mg/l as detected by the desiccator method which in the perforator test corresponds to about 8-9 mg/100 g absolutely dry particle board and thus is slightly above the limit of E₁ (Dunky 2006). Although there are still debates on the effective levels of formaldehyde emissions (Liteplo & Meek 2003, Hauptmann et al. 2003, 2004, Arts et al. 2006, Orsière et al. 2006), Japan tightened by legislation (Japanese standards JIS A 5908 2003 and JIS A 5905 2003) formaldehyde

emissions from panel boards to nearly the zero level (class F***: limit of formaldehyde emission of panel boards in interior use ≤ 0.5 mg/l as detected by the desiccator method which in the perforator test corresponds to about 4 mg/100 g absolutely dry particle board; class F****: limit of formaldehyde emission of panel boards in interior use ≤ 0.3 mg/l as detected by the desiccator method which in the perforator test corresponds to about 2 mg/100 g absolutely dry particle board; Dunky 2006, Risholm-Sundman et al. 2007). Modern production strategies target therefore at fabrication of formaldehyde emission-free boards (Dunky 2006, Roffael 2006; see also Chapters 16 and 18 of this book). The release of formaldehyde is mainly caused by two factors. It can either be due to free formaldehyde in the board that has not yet reacted or be due to formaldehyde formed by hydrolysis of the aminoplastic bond as a result of temperature and humidity. Total amounts of formaldehyde emissions from aminoplastic-bonded panel boards add together from i. gaseous formaldehyde being present in cavities of the boards, ii. formaldehyde solved in the moisture of the boards, iii. formaldehyde poorly bonded in form of methylol groups from ends or within polymeric resin chains, iv. formaldehyde poorly bonded in form of hemiacetals that is released during gradual hydrolysis of the resin taking place even at ordinary environmental conditions, v. formaldehyde bonded in methylene and ether bridges in the UF molecule that is released only under strong hydrolysis conditions (particularly at higher temperatures by an impact of a high moisture content), and vi. formaldehyde released naturally by the wood itself (Pizzi 1983, Dunky 2006, Roffael 2006). Addition of formaldehyde scavengers to the wood material in panel board production might help to reduce such formaldehyde emissions (Sellers et al. 1991, Tohmura et al. 2005, Kim et al. 2006, Miyamoto et al. 2006) - avoidance of formaldehyde as much as possible by replacing the type of glues is a most obvious strategy and another to establish techniques for production of binder-free boards. Strategies and further research projects in these areas are presented in Chapters 16 and 18 of this book.

At this place, only cement- and gypsum-bonded panel boards (cement-bonded particle- and fibreboards, gypsum-bonded particle and fibreboards) shall shortly be named. Such types of panels have unique properties making them attractive for niche applications (Jorge et al. 2004). They are characterised by high moisture resistance and dimensionally stability when subjected to water soaking (Lee 1984) and may therefore serve as cheap construction material for walls and for roofing of houses in developing countries with high temperature and humidity (Ramirez-Coretti et al. 1998, Savastano et al. 2003). Due to their high resistance to fire (Lee 1985), they can act also as a good replacement of asbestos (Soroushian et al. 1995, Savastano et al. 2004, Coutts 2005). Little is known yet on durability of cement- and gypsum-bonded boards, but they seem to have a high resistance to white-rot and brown-rot fungi (Goodell et al. 1997, Okino et al. 2004). For applications in residential constructions in climates with frost, resistance against freezing and

thawing would need to be improved (Kuder & Shah 2003). Generally for broader ranges of application, the often observed low quality bonding between cements and certain wood species or non-wood fibres due to incompatibilities between the different materials is a problem that remains to be better solved (Karade et al. 2003, Jorge et al. 2004).

Outlook

Since the beginning of larger-scale industrial production in the last century, various types of high-quality panel boards made from wood fibres, particles, strands etc. have successfully been established on the market, with local preferences for one or the other type of board. Production conditions as well as board properties have been optimised over the time, both under economical aspects as well as under the aspect to minimise or avoid environmental and health hazards during production and during later use of the products. The developments in the field are however not at an end which is quite obvious from the currently ongoing broadening of material types, material forms, material mixtures, formulations of glues, and production conditions, last but not least driven by concerns on the sustainable use of our available resources.

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16. Natural Binders

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Introduction

Long before **conventional binders** (**synthetic bonding agents** from petrochemicals, **synthetic resins**) were developed for panel board production (see Chapter 15 of this book), adhesives from renewable resources (casein, soybean protein, blood, bones, and others) were known and in use from ancient times up the middle of the last century (River et al. 1991, Keimel 2003, Frihart 2005). An **adhesive** is “*any substance that is capable of holding materials together in a functional manner by surface attachment that resists separation. Adhesive as a general term includes cement, mucilage, glue, and paste - terms that are often used interchangeably for any organic material that forms an adhesive bond*” (Encyclopedia Britannica online 2007). In the manufacture of wood-based panels with conventional synthetic resins, bonding agents cause up

to twenty and more percent of the total production costs (Deppe & Ernst 2000, Unbehaun et al. 2000; for prices in year 2006, see Table 1 in Chapter 15 of this book) and costs for conventional crude-oil-based binders are currently rising (EUWID 2007). With steadily increasing crude oil prices and increasing environmental awareness, “**natural binders**” from renewable resources (“**bio-based binders**”, “**bio-binders**”, “**bio-based adhesives**”, “**bio-adhesives**”) regained attraction as alternative bonding agents (Dunky 2004, Frihart 2005, Pizzi 2006). Even under optimised conditions (for example urea-formaldehyde ratios of 1:1.05 to 1:1.15), synthetic binders containing formaldehyde as a necessary reaction component (see Chapter 15 of this book) give rise to later emission of non-bonded formaldehyde from the pressed wood-based panels - due to that part of the formaldehyde that did not react during the hot-pressing process and due to formaldehyde that is successively over the time released from the adhesives through hydrolysis (Nemli & Öztürk 2006, Dunky 2006b, Roffaël 2006; see Chapter 15 of this book). Production of nearly emission free boards, so called E^{***} and E^{****} boards defined by the Japanese standards JIS A 5908 (2003) and JIS A5905 (2003) with formaldehyde emission values as measured by the desiccator method (Dunky 2006a,b) of ≤ 0.5 mg/l and ≤ 0.3 mg/l, respectively, can be realised by the use of non-formaldehyde bonding agents, leaving the natural emissions from wood the only possible source of formaldehyde (Meyer & Boehme 1995, 1997, Schäfer & Roffaël 1999, 2000, Manninen et al. 2002, Miyamoto et al. 2006, Roffaël 2006). With the use of natural bonding agents, the wood-based panel industries will identify themselves as environmentally and health-conscious producers. To this end, much research is currently conducted on identifying and characterising natural glues for the sustainable production of high-quality panel boards (Pizzi 2006).

Adhesives from cellulose, hemicellulose, starch, dextrans, and other carbohydrate polymers

The main groups of carbohydrates in plant waste materials are **celluloses** and **hemicelluloses**. Cellulose is made up by cellobiose units, building blocks of two β (1-4)-glycosidic linked glucose molecules (Raven et al. 2000; compare Fig. 2 in Chapter 21 of this book). Hemicelluloses are mixtures of branched carbohydrates made up by various types of monosaccharides (Ebringerová et al. 2005; see Fig. 4 to 6 and accompanying text in Chapter 17 of this book). Degradation products of carbohydrates in plant waste materials obtained by acid hydrolysis give relatively expensive dark-coloured **furanic resins** consisting of furfuraldehyde and furfuryl alcohol. Hydrolysates may further contain sugar decomposition products such as levulinic acid, and, in addition, lignin degradation products such as vanillin and homovanillic acid (e.g. see Burtscher et al. 1987, Fenske et al. 1998, Kline et al. 2002, Dinardo & Larson 1994). Furanic resins using the plant waste degradation products furfuraldehyde and furfuryl alcohol as adhesives' building blocks are

however considered synthetic. They can principally be applied in panel board production although care has to be taken by the toxic character of furfuryl alcohol before a bonding reaction takes place (Belgacem & Gandini 2003, Pizzi 2006). Phenolated acid liquified lignocellulosic and cellulosic plant waste material including liquified wood has lately found some attention as bio-adhesives. During acid liquefaction, cellulosic components lose their pyranose ring structure and become phenolated. The phenolated products provide phenolic groups mediating good wood adhesive properties (Alma & Shiraishi 1998, Alma et al. 1998, Alma & Acemioglu 2004, Alma & Bastürk 2006; see below). Substitution of portions of commercial phenol-formaldehyde (PF) resins up to 55% by addition of a variety of carbohydrates - from glucose to polymorphic hemicelluloses - is also possible. High proportions of the carbohydrates co-react with the phenol in these mixed binders containing in addition low amounts of urea and formaldehydes. Successful industrial trials with such mixed binders have been reported (Trosa & Pizzi 1998, Pizzi 2006).

Of special interest are polymeric carbohydrates with natural adhesion properties. **Starch** as a polymeric carbohydrate is produced by plants as a way to store the chemical energy assimilated during photosynthesis. It is a mixture of the two natural polymers, amylose and amylopectin, both build up by α -glucose units (compare Fig. 2 in Chapter 21 of this book). In amylose, 1000 and more glucose monomers link to each other in head-to-tail arrangement by $\alpha 1 \rightarrow 4$ linkages. In amylose, there is little or no branching and, therefore, the linear polymer adopts a helical structure. In contrast, the coiled amylopectin has branches at every 12th to 25th of the $\alpha 1 \rightarrow 4$ linked glucoses which connect by $\alpha 1 \rightarrow 6$ linkages to the respective monomers in the main-chain of the molecule (Raven et al. 2000). Of all renewable agricultural and forestal primary products (includes wood, cellulose, oil, sugar, starch, etc.), starch is after fats and oil the third-most used resource in the chemical industry (Sarathi Reddy & Basappa 1993, Warvel 1998). Starch is found primarily in the seeds, fruits, tubers, and pith of stems of plants, most notably in corn, wheat, rice, sago, and potatoes. Starch is one of the most abundant natural polymers, relatively inexpensive, and therefore an interesting material for fibre bonding (Baumann & Conner 1994, Imam et al. 1999, Clare et al. 2002, Pan et al. 2005). A large quantity of industrial starch is used in the paper industry as filler, pigment material, and adhesive to bond vessel segments and loose surface fibres in order to enhance paper strength and stiffness (Maurer 1998, Brouwer et al. 2000, 2002, Höpke 2002). Furthermore, it is used as adhesive in manufacturing of corrugated boards (Borchers et al. 1993, Höpke 2002). Starch is also added as an extender to various types of conventional adhesives in order to enhance their environmental performance by reducing the absolute usage of synthetic resins and, in consequence, thereby also the emissions of toxic formaldehyde as well as the production costs (Basta et al. 2005, 2006, Müller 2005, Pan et al. 2006; see below). Moreover, starch may act as a scavenger for free formaldehyde (Basta et al. 2006).

Starch yields adhesives with excellent affinity for polar materials such as cellulose. Starch-based adhesives wet the polar surface of cellulose, penetrate crevices and pores, and thus form strong adhesive bonds. Bonding is the result of both, mechanical interlocking and van der Waals forces (Imam et al. 1999). Most starches consist of about 70% amylopectin and 30% of hot water soluble amylose. However, there are source-dependent differences and these will influence the properties of the starch as adhesive (Zeppenfeld 1991, Eriksson et al. 2005). In spite of this, a common problem with starch-based adhesives is a reduced water-resistance of the products (Basta et al. 2005, 2006, Dix 2006; see below).

Dextrins are mixtures of short carbohydrate polymers that are obtained from starch by acid hydrolysis. In contrast to starch, dextrins are easier to solve in water. They have a low viscosity, a higher solid content in solutions, and better bonding qualities (Kennedy 1989, Clare et al. 2002). Other adhesive, non-toxic, and biodegradable carbohydrate polymers come from bacteria and fungi, for example **glucans** (pollulan) from higher basidiomycetes (Haars & Kharazipour 1998) and **exopolysaccharides (EPS)** from marine bacteria characterised by a special high water and low temperature resistance (Labare et al. 1989, Weiner 1997). Qualities of bacterial exopolysaccharides provide to glued panels differ with the bacterial source they come from (Haag et al. 2004, 2006). The anaerobic gram-negative bacteria *Ruminococcus albus* and *Clostridium thermocellum* degrade plant wastes by a range of cellulolytic enzymes, a property that is used in bio-ethanol production. Fermentation residues from bio-ethanol production consisting of incompletely fermented fibre and attached bacterial cells with adhesive carbohydrate material in the surrounding glycocalyx (a polysaccharide mucilage layer around the cells) has successfully been tested in plywood manufacture (Weimer et al. 2003, 2005). Hydrocolloidal **glucomannans** (a polysaccharide of β 1-4 linked D-glucose and D-mannose) from tubers of the Araceae *Amorphophallus konjac*, **chitosan** [β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine] as a deacylated product of crustacean **chitin** (a polymer made of β -(1-4)-linked N-acetyl-D-glucosamine units) and mixed combinations of these gave promising results when tested in plywood gluing. Glucomannan-chitosan mixtures performed better in terms of adhesiveness and bonding properties than casein and soybean glues (for more information of these types of natural binders see below) and chitosan was superior to conventional **urea-formaldehyde adhesives (UF resins; see Chapter 15 of this book)** with respect of dry bond strength (Umemura et al. 2003). Improved composite performance was reported upon application of chitin and chitosan as coupling agent between the hydrophilic surfaces of wood flour and the hydrophobic polymer [PVC, poly(vinyl chloride)] matrix in the production of wood-plastic composites (Shah & Matuana 2005).

Starch, dextrins and other types of poly-carbohydrates mentioned above that possess some own adhesion properties are usually not applied as sole adhesives in manufacturing of derived timber products but are added as **extenders** to conven-

tional resins in order to reduce the required amount of the petrochemical-based glues. Starch (native or modified) with conventional phenol-, melamine- or urea-formaldehyde resins, bacterial EPS with **phenol-formaldehyde (PF) resin** and mixed composites of starch and tannin (see below) can even provide better board properties since the positive features of the different co-adhesives are combined (Conner et al. 1989, Dix 1987, 2006, Dix & Marutzky 1988, Johnson & Kamke 1994, Imam et al. 1999, 2001, Turunen et al. 2003, Basta et al. 2005, Weimer et al. 2005). Combinations of native, unmodified starch with UF resins are however unsuitable (Plath 1972). A new development is an adhesive for plywood which is prepared by crosslinking starch and a hydroxyl functionalised polymer (polyvinyl-alcohol, PVOH) with hexamethoxymethylmelamine (Cymel 323) in the presence of citric acid as a catalyst. To increase moisture resistance, **hydrophobisers** such as latex were added to the formulations. At the same time, physical properties of panel boards were optimised (Imam et al. 1999, 2001). Furthermore, starch and other poly-carbohydrates might be mixed with other bio-binders (e.g. lignin and tannin) in order for them to support each other in their positive properties (Haars & Kharazipour 1998, Dix et al. 1998a, see below). However, incompatibilities between different types of bio-binders can occur. For example, flocculation was observed when mixing fungal glucans with certain types of lignin (Haars & Kharazipour 1998).

For application as wood adhesives, carbohydrates do not need to be of pure form. Adequately processed starch-containing agricultural waste materials such as potato-pulp (Kharazipour & Bergmann 1998, Müller 2005; see below) and commercial rice bran (Pan et al. 2005, 2006) have successfully been tested as effective bio-based adhesives. The starch and also the protein (see below) in heat- and alkali-modified rice bran reacts adhesive. In test trials with particle boards, up to 30% of the synthetic **PMDI (polymeric methylene diphenyl diisocyanate, see Chapter 15 of this book)** could be replaced with heat- and alkali-modified rice bran to yield products with properties similar to those of PMDI-bond particle board (Pan et al. 2005, 2006).

Mechanically-enzymatically decomposed potato-pulp in production of medium density fibreboards

The most important suppliers of starch in Europe are wheat, corn and potatoes. As an example in the year 2000/2001, the production of potatoes for the industrial use in Germany amounted to 5.5 million tonnes. Of these, 2.9 million tonnes were utilised for starch production and 45% of the produced starch was used in non-food industries: 18% in chemical industries (e.g. for washing powders, cosmetics, glues, films and building materials), 21% in the paper industry, and 6% in cardboard production (bioSicherheit 2007). During starch production from potatoes, huge amounts of waste accumulate. The potato-liquor (or potato-juice, i.e.

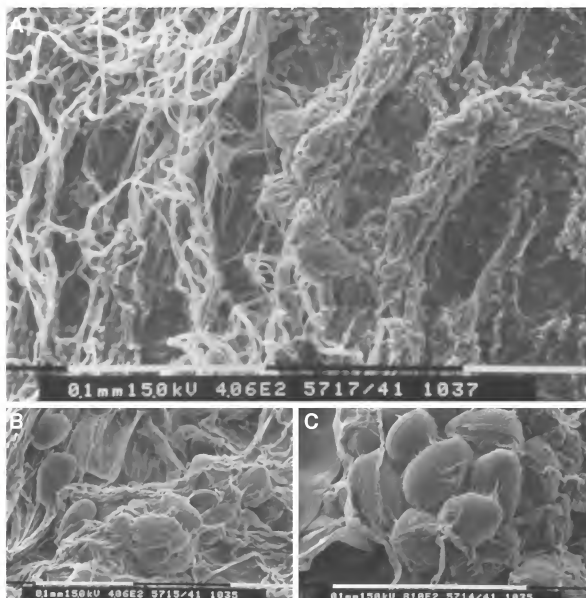


Fig. 1 Scanning electron microscope (SEM) pictures of potato pulp showing cell wall relicts (A), discrete starch grains in between cell wall debris (B), and nests of intact starch cells (C). After Kharazipour & Bergmann (1998)

the fluid left from the extraction of the starch granules from torn apart tuber cells) and the **potato-pulp** (i.e. a by-product consisting of parenchymatic tissue and 95% water; Fig. 1) have the largest part in it. Starch production of one ton potatoes gives rise to 235 kg potato-pulp. For its disposal, it is currently used as feeding stuff after costly drying. However, any other kind of industrial application could be more economic for the starch industry (Olson 2000). Potato-pulp for example might be applied in form of binders in wood composite production (Kharazipour & Bergmann 1998). Due to a >30% content of residual starch in the

dry matter (Mayer & Hillebrandt 1997; Fig. 1) and due to specific cell wall components of parenchymatic tissues - in particular **pectin** (Fig. 2) - potato-pulp shows excellent adhesive properties (Mayer 1998). Up to 17% of the dry matter of the potato tissue is pectin (Mayer & Hillebrandt 1997) which has good water solubility and a high ability of swelling (Jarvis 1984, Ryden et al. 2000, Zsivanovits et al. 2004).

Pectins are linear mixed polymers of α -D-galacturonic acid units (Fig. 2) linked by $\alpha 1 \rightarrow 4$ glycosidic linkages. Depending on the pectin source and the extraction mode, carboxyl groups are partially esterified with methanol (Fig. 2) and, in certain pectins, hydroxyl groups are partially acetylated. At acidic pHs, hydrogen bonding may occur between pectin chains via free hydroxyl groups at the galacturonic acid units. Pectin-water hydrogen bonds compete with these pectin-pectin interactions. Thus, with increasing water content, the overall pectin bonding decreases. Neutral sugars such as galactose, glucose, rhamnose, arabinose, and xylose (usually in weights of about 5-10% of the amounts of α -D-galacturonic acid) are often present in pectin preparations, either as side-chains to the galacturonic main chain or, more likely, as a part of contaminating polysaccharides (glucans and xyloglucans). These sugars can act as dehydrating agents and, thereby, support pectin adhesion (Rolin & De Vries 1990, Turquois et al. 1999).

Hydrogen bonds cause not only that pectin molecules stick together but they are also the basis for the bonding capacity of starch (Vilaseca et al. 2007). Hydrogen bonds mediated by free hydroxyl groups of both pectin and starch are relevant for the function of potato-pulp as adhesive. An adhesive effect in wood composite production is caused when such free hydroxyl groups in pectin and starch interact with the surface of wood fibres (Müller 2005).

The performance of potato-pulp as an adhesive has been tested in medium density fibreboard (MDF) production (Fig. 3). In summary, the main results were: i. potato-pulp *per se* does not mediate an adequate bonding capacity for the production of wood-based composites. ii. potato-pulp needs to be enzymatically decomposed in order to release those cell wall components that act adhesive. iii. enzymatic decomposition is alleviated by mechanical pre-disintegration (Kharazipour et al. 1993, 1994, Kharazipour & Bergmann 1998, Müller 2005).

At the laboratory scale, mechanical disintegration of potato-pulp can take place by a cutter which is normally used by butchers for mincing meat (Kharazipour et al. 1993, Kharazipour & Bergmann 1998, Müller 2005). Working with high speed, the switchblades of the cutter break up the walls of those cells that are found still intact in the potato-pulp (Fig. 1C). Subsequently to mechanical disintegration, pectin is hydrolised by pectinases into its basic compounds in order to release the starch encased in the pulp, to free cell-bound water, and to decrease the viscosity of the pulp for easier mixing the binder with wood chips and fibres (Kharazipour et al. 1994, Kharazipour & Bergmann 1998, Stroot 2001, Müller

2005). Pectinases represent a heterogeneous group of pectolytic enzymes (esterases, glycosidases, and lyases) that differ in the mode of operation and points of

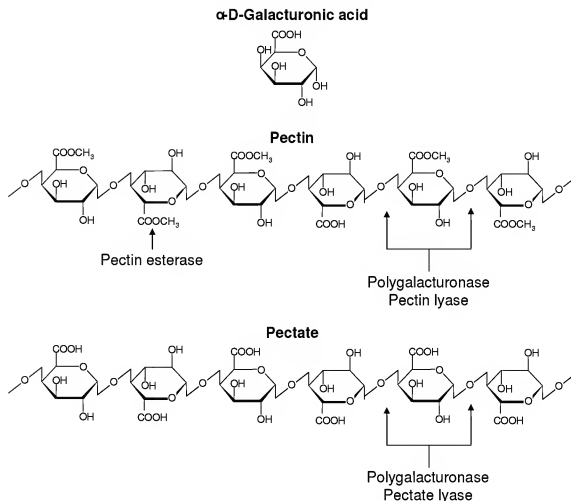


Fig. 2 Structures of α -D-galacturonic acid, pectin, and pectate, and enzymatic actions on the biopolymers. Pectinesterases (pectin demethoxylase, pectase; E.C.3.1.1.11) catalyse the hydrolysis of methylester groups of pectins to give methanol and non-methoxylated pectates. Polygalacturonases (endopolygalacturonase, pectinase, pectin depolymerase; E.C.3.2.1.15) mediate random hydrolysis of 1,4- α -D-galactosiduronic linkages in the homogalacturan backbones of the biopolymers. Pectin lyases (pectin methyltranseliminase, polymethylgalacturonic transeliminase, endo-polymethylgalacturonic transeliminase; E.C.4.2.2.10) and pectate lyases (pectate transeliminase, polygalacturonic transeliminase, endogalacturonate transeliminase; E.C.4.2.2.2) cleave α -1,4 links between galacturonosyl residues in the homogalacturan backbones of the substrates via a β -elimination reaction resulting in the formation of an unsaturated C4-C5 bond at the non-reducing ends of the cleaved polysaccharides. Pectin lyases are specific for highly methylated substrates whereas pectate lyases act on demethylated or low-esterified forms of pectin (Deuel & Stutz 1958, Rombouts & Pilnik 1978, Mayans et al. 1997)

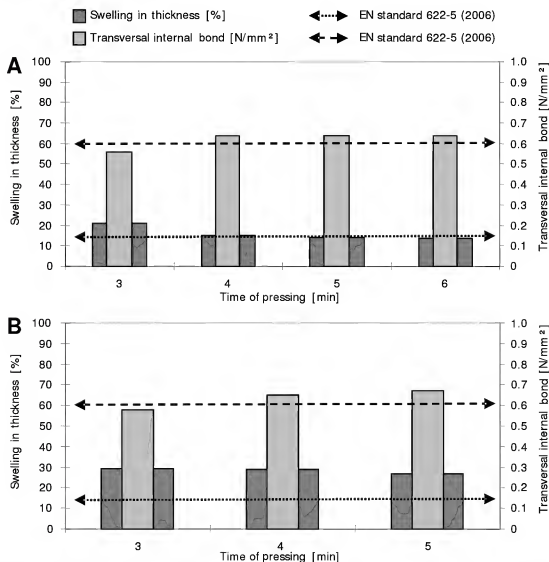


Fig. 3 Swelling in thickness after 24 h in water and transversal internal bond strength of medium density fibreboard (MDF). A. MDF produced with 100% mechanically-enzymatically decomposed potato-pulp, 2% keying agent, and 2% hydrophobic wax (relative to absolute dry fibre). B. MDF produced with a 1:1 mixture of urea-formaldehyde resin and mechanically-enzymatically decomposed potato-pulp (relative to absolute dry fibre). Properties were tested on the basis of European standard EN 622-5 (2006)

attack in the decomposition of pectin (Fig. 2). As the result of the enzymatic actions, long-chain pectin molecules will be split into smaller oligomers of α -D-galacturonic acid units (Jayani et al. 2005). Mixtures of pectolytic and other enzymes are commercially available for the use in food, textile, and paper industries (Kashyap et al. 2001). Of various enzyme preparations tested in the conditioning of potato-pulp for MDF production, a crude mixture of polygalacturonases [poly(1,4- α -D-galacturonide) glycanohydrolase; EC 3.2.1.15] and cellulases

(EC 3.2.1.4) produced by *Aspergillus aculeatus*, *Aspergillus niger*, and *Trichoderma reesei* proved to be most effective in the enzymatic decomposition of mechanically disintegrated potato-pulp. The treatment with the polygalacturonases is thought to cause an increase in adhesiveness of the potato-pulp whilst cellulases help by disintegration of the cellulose in the cell walls in the potato-pulp (Kharazipour & Bergmann 1998, Müller 2005). In laboratory trials, after 4 hours incubation with a 10% (w/w) solution of the enzymes, mechanically-enzymatically decomposed potato-pulp can be dewatered by spray-drying for later use. Alternatively, enzyme-treated potato-pulp can directly be applied as adhesive in manufacturing of MDF by spraying the fibres. In the latter case, drying of the pulp proceeds during the production process of the fibreboards (Kharazipour & Bergmann 1998, Müller 2005).

MDFs were made on the pilot plant scale (see the MDF pilot plant in the "Biotechnikum" of the Institute of Forest Botany in Göttingen in Fig. 16 to 18 in Chapter 15 of this book) with 15% enzyme-treated potato-pulp per weight dry fibre at a density of 800 kg/m³ (Müller 2005). However, such fibreboards did not reach the required qualities specified by the European standard EN 622-5 (2006). Especially the swelling values in water were unacceptable. Hydrophobic wax (1-2%) and **primer** (1-2%) were added in order to decrease swelling and to increase board strengths. The best results were achieved upon addition of 2% hydrophobic wax and 2% of the primer (coupling agent) promoting better contacts between fibres and the mixed potato-pulp-UF binder (Fig. 3A). In further test series, the decomposed potato-pulp was mixed with UF resin (0-50%) in order to increase cohesiveness. Fibreboards produced with a 1:1 mixture of enzyme-treated potato-pulp and UF resin reached acceptable values for transverse tensile strength (Fig. 3B). Time of pressing was found to play an important role. Fibreboards pressed for less than 4 or 5 minutes had inferior physical and technological properties than fibreboards pressed for longer time (Fig. 3). Overall, these results at the pilot scale corroborate that potato-pulp is a possible alternative to substitute at least parts of conventional synthetic resin. Still, this outcome will have to be confirmed at an industrial scale. An exclusive use of potato-pulp is however doubtful. Further to UF resins, mixtures with other synthetic resins should also be designed and tested in order to exploit their physiological and technological properties in an ecological and economical best optimal way. In addition to MDF, the broad range of other types of timber-derived panel products (see Chapter 15 of this book) offers a variety of materials and methods of production in which potato-pulp-based adhesives might be applied (Müller 2005).

Proteins as adhesives

Proteins are macromolecules composed of 20 different amino acids linked in chains via peptide-bonds. Amino acids may have an acidic, a basic, or a neutral

character. Physical and chemical properties of proteins are determined by the abundance and order of the different amino acids in the polypeptide chains as well as by the individual three-dimensional structures proteins adopt. Because of the amphoteric character of the amino acids possessing at least one carboxyl group (-COOH) and at least one amino group (-NH₂), proteins are ampholytes that, depending on their environment, may react as either an acid or as a base (Raven et al. 2000). For application as binders, naturally soluble proteins are required that can transform into an insoluble stage. Protein denaturation, the transformation into an insoluble form, might be caused by thermal exposure or by reactions with a chemical compound. Proteins offer many advantages that qualify them as possible adhesives for industrial applications – not only for the wood-based panel industry. Unlike petrochemical-based bonding agents, usage of amphoteric proteins as adhesives in panel board production does not depend on a certain critical pH-value. In consequence, protein-based adhesives can be combined with many hydrophobic substances, fungicides, flame retardants, and other additives. Proteins are renewable resources obtained in large quantities from animals and plants by husbandry and fishery – often also in form of waste products. Proteins are all-year around available and, compared to conventional bonding agents, the price of various kinds of proteins is low and production does not depend as such on the non-renewable resource crude oil. The four major groups of cheap and abundantly available proteins are the water-soluble animal **albumins** such as blood albumins and egg white proteins, the long fibrous **collagens** from animal connective tissues, skins, and bones, the **caseins** as main milk proteins, and **plant proteins** from cereals, soybeans, etc. (Krug 2002, Krug & Heep 2006, Schöpfer 2006). Collagens named after the Greek word “kolla” for glue is the oldest type of glue and was used by humans already more than 8,000 years ago (Walker 1998).

Proteins in form of blood and from fish, casein, cereals, and soybean were established as adhesives already in the earliest days of production of wood-based panels (Hubbard 1887, Weakley et al. 1963, Weakley & Mehlretter 1965, Spielman 1986, Liu 1997). Particularly in the production of plywood, soybean protein has massively been used between the 1930s to the 1960s (Liu 1997). However, mainly from the 1940s synthetic resins such as UF and PF resins rapidly replaced the bio-adhesives by superior durabilities, viscosities, and pot lives, and, finally, by a better price (Liu 1997; see Chapter 15 of this book). Nevertheless, an interest in binders on protein basis revived and bio-binders could become fully competitive if economical solutions to their technical drawbacks would be found (Kumar et al. 2002). Different kinds of animal and vegetable proteins in different binder formulations and different applications are in recent time therefore tested in their potentials as bonding agents on laboratory, pilot, and eventually also industrial scale production.

In the early 1960s, Weakley & Mehlretter (1965) for example reported the development of an adhesive mixture consisting of soybean protein and blood to be

used for the hot pressing process during the production of pine veneers. Amoldt (1964) in contrast studied the applicability of inferior grain flour as an extender in urea-formaldehyde resins in the production of particle boards. Later, Dix and colleagues tested the ability of different cereal flours to stretch polymer diisocyanates (Dix & Marutsky 1988, Roffael & Dix 1992). Polymers in wheat flour have been shown to form entanglement networks with the thermosetting synthetic resins which survive hot-pressing and resin hardening and can contribute to the final quality of adhesion (Kamoun et al. 1998). Next to other ingredients, cereal flours contain storage proteins and starch, wheat for example about 10 to 14% proteins and 60 to 65% starch (Shewry & Tatham 1990, Franke 1997). Both types of polymers will likely contribute to the adhesive character of the flours (Weakley et al. 1971, Pan et al. 2005; see above). When cereals are used for starch or glucose syrup production, the storage proteins accumulate as a by-product in the watery milling solutions (Lawton 2002, Schöpfer 2006). The solids including the proteins can be concentrated to levels above 40% by evaporation of the liquid. Such protein concentrates are excellent raw materials for natural binders and come already from starch companies (e.g. from Cerestra, Vilvoorde, Belgium and Barby, Germany) with an optimal viscosity for spraying as glue onto fibre material. Moreover, they are abundantly available at cheap price - the price of a ton of wheat protein solution (about 300 € in the year 2005) is below the price of a ton of conventional UF resin (about 360 € in the year 2005). In Germany, 1.2 million tons of wheat are transformed per year to starch and starch derivatives. Per ton of wheat, 140 kg proteins are left which adds up to a total of 168,000 tons (Schöpfer 2006).

Wheat protein concentrates have been tested in MDF production on the pilot scale in the "Biotechnikum" of the Institute of Forest Botany in Göttingen (see Fig. 16 to 18 in Chapter 15 of this book for views of the MDF pilot plant). Without any problems, established machines and standard parameters of the dry processes of MDF production can be applied with the wheat protein bio-binder. Both, mixtures with synthetic binders (UF and PF resins; tested rates of protein binder to synthetic binder were 0% : 100%, 25% : 75%, 50% : 50%, 100% : 0%) and wheat proteins as pure binders were tested with mostly satisfying results. The mechanical properties of most boards produced on the pilot scale with wheat protein concentrate fulfilled the recognised norms. Likely due to the hydrophilic character of the proteins, a high water absorption behaviour and swelling of boards become however problematically when the wheat proteins in the binders reach rates of 50% and above. Negative was the observed incompatibility between wheat protein concentrates and conventional hydrophobisers frequently applied in wood panel productions. In future research, the optimisation of the swelling behaviour of mainly or purely wheat protein-bonded MDF needs further attention. Favourable were the very low formaldehyde emissions of boards bonded with high rates of wheat proteins - the bio-binder appears to act as formaldehyde scavenger. By their good biodegradability, wheat protein-bonded MDF can easily

and environmentally friendly be composted if required for disposal after use (Schöpper 2006, Schöpper & Kharazipour 2006).

The typical wheat protein solution from starch production had a pH around 4.5 and contained 20-30% proteins, 30-40% hemicelluloses, and 40-50% saccharides. The glass transition temperature of the wheat protein adhesive is at about 140°C. Preliminary analytical results suggest that at this pressing temperature the bonding mechanism of the wheat protein adhesive occurs via generation of irreversible bonds between proteins and sugar residues (Schöpper 2006).

Compared to other plant proteins (soybean, maize, and rapeseed), bonding with wheat protein gave best results in test series of particle board production performed by Krug and colleagues. Binder formulations of wheat protein were judged equal to formulations of soybean protein in solubility and swelling but technical properties of pressed boards were of better quality when using soybean proteins. A problem was the moisture resistance of boards not allowing applications under humid conditions. Mixed binder formulations of PF resin replaced by 25% of soy proteins in contrast gave the necessary low values in swelling (Krug & Sirch 1999, Krug 2002, Krug & Heep 2006). The same group showed in MDF production that PF resins blended with soybean proteins can result in better transverse tensile strength and reduced thickness swelling compared to pure PF resins (Krug 2002). Similar tendencies were encountered in production of OSBs with soybean-blended PF resins. Protein-bonded OSBs were in addition more attractive by a lighter colour (Krug 2002, Krug & Heep 2006).

Animal proteins accumulate in much lower amounts than some of the plant proteins. About 110,000 tons of collagens, 30,000 tons of casein, and 10,000 tons of whey proteins are estimated to be at disposal every year in the European Union (Krug & Heep 2006). Traditional protein-based glues from animal origin had a high viscosity (Golick & Dike 1941, Ash & Lumbuth 1954, Weakley & Mehlretter 1964) and could be used for plywood gluing but not for spray application in the manufacture of particle boards, fibreboards, and OSB. Formulations of PF mixed with hydrolysed blood protein have now been created that perform comparable to PF resins. MDF produced with the glue met the requirements for exterior use. Plant proteins (soybean protein, peanut flour) were also tested in mixtures with PF but soybean-bonded boards met requirements only for indoor use whilst peanut flour-bonded boards failed even some of these (Yang et al. 2006). Wang & Pizzi (1997) tested egg albumin and collagen in mixtures with UF adhesives for plywood bonding. Functional groups in the proteins reacted with the formaldehyde and the reactive methylol groups of the UF resin into a protein/UF gel that gave high water resistance to glued plywood. With the increasing economical, ecological, and health concerns on usage of synthetic binders and formaldehyde, a strong focus in research is however on development of pure bio-based binders, thereby making better use of underexploited waste materials available from agriculture and food industries.

Soybean protein as a well established and thoroughly used binder in the past (Liu 1997) is nowadays again an attractive candidate for usage as a wood composite binder since it is available each year in huge amounts at cheap prices. Soybeans contain 30-50% of protein (Nielsen 1985, Garcia et al. 1997) and the world production for example just in the year 2006 was over 221 million tons (FAOSTAT 2007). Soybean proteins have repeatedly been investigated in production of different types of panel boards. Pressing conditions very much appear to determine adhesive strength. Both, too high and too low pressure, result in poor bonding by different reasons (probably damage to the wood surface and lack of bond formation, respectively) Cheng & Sun 2006). One of the major reoccurring drawbacks of soybean protein application as adhesives in panel board production is the relatively poor water resistance (Kuo et al. 1998, Wescott et al. 2006). Mixing with hydrophobisers can help (Li, K.C. et al. 2004). Alkali-modification of the protein has repeatedly been reported to increase adhesive strengths and mechanical properties of particle- and fibreboards (Hettiarachchy et al. 1995, Kalapathy et al. 1996, Wang & Sun 2002, Leiva et al. 2007, Lorenz et al. 2007). Modifications by proteolytic enzymes (e.g. papain or trypsin) and by urease to improve binder viscosity went along with and an increase in adhesive strength (Hettiarachchy et al. 1995, Kalapathy et al. 1995, Kumar et al. 2004). Increase in both adhesive strength and hydrophobicity of soybean protein were also reported upon moderate esterification of free carboxyl groups of the proteins with ethanol (Wang, Y. et al. 2006) and upon urea treatment (Sun & Bian 1999, Huang & Sun 2000a). Two of the five major soybean proteins [conglycinin (7S) and glycinin (11S) making up about 80% of the soybean storage protein; Garcia et al. 1997] were shown to provide a better adhesion when treated with urea. Very interestingly, the two storage proteins differed in adhesion performance with the types of wood tested: urea-modification increased adhesion of conglycinin on cherry and walnut whilst glycinin by urea-modification obtained a better adhesion on pine (Zhang & Hua 2007). Different soybean cultivars vary in the relative amounts of these proteins with some having mostly conglycinin and some having mostly glycinin (Liu et al. 2007) and there are major variations particularly in the acidic subunits of glycinins from different cultivars (Natajaran et al. 2007). The source of the soybean protein must thus be expected in applications to influence binders' properties. Not surprisingly therefore, proteins from different genetic lines were reported to perform differentially in gluing wood (Stoll et al. 2006). Amongst other successful attempts to increase hydrophobicity of soybean proteins for better performance in wood adhesives were treatments with sodium sulphite to dissolve protein disulfide bonds (Kalapathy et al. 1997), glutaraldehyde for crosslinking amino groups (Wang et al. 2007), treatment with compounds such as sodium dedecyl sulfate, sodium dodecylbenzene sulfonate, and guanidine hydrochloride for protein denaturation (Huang & Sun 2000a,b, Zhong et al. 2001, 2003), and polyamide-epichlorohydrin and polyethylenimine for complexation with the protein (Liu & Li 2007, Zhong et al.

2007). These various treatments to make soybean proteins water-resistant target generally at changing protein conformations and structures and at decreasing the number of functional groups on the proteins. However, sometimes with an increase in water-resistance the mechanical performances of glued boards decreased - likely by the loss of functional groups. Such negative effects might be overcome by adopting new pressing parameters and other production conditions (Zhong et al. 2002, 2003). Generally, the cheapest, most save, and most easy to apply solution to the problem has to be defined. Trying to understand soybean protein properties and how to best expose the proteins' functional groups is expected to lead to predictions on resin performance (Lorenz et al. 2007).

Biomimetics (bionics, bionical creativity engineering) investigates biological structures and processes for their use as models for the development of artificial systems and applies methods and systems found in nature to the study and design of engineering systems and modern technology (Teeri et al. 2007; see Chapter 11 of this book). Bionic approaches are now successfully used in adhesive design. Water-resistant, difficult to degrade marine mussel proteins from the feet of the animals are amongst the natural proteins with the strongest adhesive forces. Even in wet condition, they bind to inorganic and organic surfaces on which other adhesives function poorly or fail. The mussel proteins contain the unusual amino acid 3,4-dihydroxy-L-phenylalanine (DOPA) obtained by posttranslational modification of tyrosine. DOPA undergoes oxidation for high strength irreversible covalent bond formation to an organic surface whereas for binding to metallic compounds, the DOPA residues in the proteins must not be oxidised. Metal ions help the proteins to attach to inorganic surfaces and in cross-linking the proteins (Lee et al. 2006, Lauren & Wilker 2007). A special geometry mediated by their unusual amino acid composition is then thought to cause by physical interactions their impressive adhesion forces (Lin et al. 2007). For an industrial implementation on pure basis, these proteins will likely never be cheap enough unless recombinant overproduction in easy to handle biological systems becomes possible (Hwang et al. 2007a,b). However, with the start of understanding as how these adhesive mussel proteins work (Lin et al. 2007), glue proteins from other sources, e.g. from gecko food pads and marine bacteria, are studied (Lee et al. 2007b, Urishida et al. 2007). Mixed glues combining the special adhesive properties from the mussel proteins and the strong adhesion as well as excellent reusability of gecko proteins catch these days the imagination of the chemical designers as they are opening up a new dimension in adhesive techniques (Barnes 2007, Hadlington 2007, Laduc 2007, Lee et al. 2007b, Majumber et al. 2007). Stimulated by the mussel proteins, dip-coating of various types of organic and inorganic material in DOPA solutions was shown to give self-assembled surface-adherent polydopamine films ready for secondary surface reactions (Lee et al. 2007a). Amongst such secondary surface reactions could be the gluing together of two surfaces. Furthermore, synthetic polypeptides containing DOPA can be produced showing adhesive properties (Yu

& Deming 1998). For optimising protein-based wood adhesives, mussel proteins served as a model to design DOPA-grafted soybean proteins. This modification converted soybean protein into a strong and water-resistant glue (Liu & Li 2002). Increasing the free mercapto-group content in the protein as another feature known from the mussel proteins improved also the strength and the water-resistance of wood composites bonded with the designer glue of soybean protein basis (Liu & Li 2004).

Tannins

Tannins are natural **polyphenols** that divide into two classes of chemical compounds of mainly phenolic nature: hydrolysable tannins and condensed tannins. Hydrolysable tannins consist of polyol carbohydrates such as glucose whose hydroxy groups are partially or totally esterified with phenol groups such as gallic acid or ellagic acid. Condensed tannins (proanthocyanidins) are polymers of 2 to 50 (or more) flavonoid units joined by carbon-carbon bonds which are not susceptible to being cleaved by hydrolysis (Pizzi 2000, 2003, 2006). Hydrolysable tannins have been successfully used as partial substitutes (up to 50%) of phenol in the manufacture of phenol-formaldehyde resins (Kulvik 1976, 1977). However, the naturally low macromolecular structure, the low level of phenol substitution they allow, their low nucleophilicity, the limited worldwide production, and a relative high price makes them less interesting compared to the condensed tannins (Pizzi 2000, 2003, 2006).

Condensed tannins with a yearly production of 200,000 tons make up more than 90% of the world's commercial production (Pizzi 2003, Pizzi 2006). These tannins are extracted from bark, wood or fruits, for example from *Mimosa tenuiflora*, *Quebracho* species, *Acacia mearnsii*, and some kinds of pines and spruces. The most important nations, which produce derived timber products (especially particle boards) with tannin-formaldehyde resins are Argentina, Chile, South Africa, and Australia (Kharazipour 1996, Li & Maplesden 1998, Rosamah 2003). Tannins are obtained with efficiencies of 22 to 48% of the dry mass from barks of tropical trees by hot water extraction whilst rates of yields from barks of conifers in temperate zones are with 14 to 16% lower by the high fraction of water-insoluble phlobaphenes (Dix & Marutzy 1983, Dix et al. 1988b).

Tannins in industry are applied in resins classically in combination with formaldehyde functioning as a hardener (Pizzi 1980, 2003, Roffael et al. 2002). At certain pHs, tannins and formaldehyde transform together into high-molecular branched condensation-products that are poorly hydrolysable (Pizzi 1977, Pizzi & Roux 1978, Roussow et al. 1980, Prasetya & Roffael 1991, Garnier et al. 2002). Tannins of different sources (from different tree species, different habitats, etc.) differ in chemical structure and require therefore different amounts of formaldehyde to start the condensation reactions (Prasetya & Roffael 1991, Dix et al.

1998b, Tahir et al. 2002, Rosamah 2003, Hashida et al. 2006, Pizzi 2006). The most predominant factor is nevertheless the pH that must be alkaline in the range of around pH 10 (Garnier et al. 2002).

In Australia in 1995, about 6,200 tons of tannins with an estimated value of over \$A 9 million were used in wood adhesives (Doran 1995). In Germany, particle boards were made with *Quebracho* and *Acacia* tannin-formaldehyde resins in a small-sized industrial scale starting from 1995. These particle boards, known as "Schlingmann-Natura", have extremely low formaldehyde evaporation (Roffael et al. 2001). Furthermore, tannin-formaldehyde resins are employed for bonding of MDF. Here, *Quebracho* tannins can partially be replaced by tannins from spruce (Dix et al. 1998b, Roffael et al. 2000). Sap- and heartwood of pine and larch were shown to perform differently well in gluing with tannin resins, particularly in MDF. The presence of extractives from heartwood increased the gelation time of the resin but all final properties of the boards were better including the level of formaldehyde release (Dix & Roffael 1995, 1997, Roffael & Dix 1997). Using condensed tannins as a binder-component is ecological and economical advantageous: the environmentally friendly tannin-formaldehyde resins are amongst the cheapest binders, generate humidity-resistant bonds, and cause low formaldehyde emission (Dix & Roffael 1997, Pizzi et al. 1997, Pizzi 2000, Rosamah 2003). Especially condensed tannins with a high ratio of pyrogallol-type B-rings have a high formaldehyde scavenging ability (Hashida et al. 2006).

Whilst tannins are long established on the market in production of panel boards for specific uses, there is much potential to broaden their application by altering tannin sources, resin recipes and production conditions, and by producing boards of new specifications (Pizzi 2000, Simon & Pizzi 2002, Dunky & Niemz 2002, Dix & Schneider 2006). Tannins can be mixed with all types of synthetic binders (Pizzi 1982, Dix & Marutzky 1985, Long 1991, Kehr et al. 1994, Zhao et al. 1995, Grigoriou 1997, Grisby & Warnes 2004) but some of the adhesive blends are inferior to conventional resins (e.g. see Kehr et al. 1994, Grisby & Warnes 2004). MDF produced with tannins replacing parts of phenol in phenol-urea-formaldehyde resins or even with 100% tannin resin can meet interior grade specifications but usually not exterior grade specifications (Roffael et al. 2000, López-Suevos & Riedl 2003). Analysis of tannin-bound *Eucalyptus* plywood revealed an increase in wettability due to the surfactant character of tannins, leading to fractures at the glue lines and a high wood failure under exterior conditions (Vázquez et al. 2003). In other cases, tannin-bound *Eucalyptus* plywood fulfilled however the norms for exterior use (Vázquez et al. 2002). Impregnating wood chips with *Pinus* bark extractives has been found to improve decay resistance whilst mechanical properties decreased. Of the tested conditions, only particle boards made from 1% bark extractives still met the specifications of boards for general purpose (Nemli et al. 2006). Laboratory trials are ongoing to further establish tannin resins for strandboard production together with the best

reaction conditions and best tannin types (Sellers & Miller 2004). Newer developments concentrate on developing resins totally free of formaldehyde by combining tannins with other bio-based material, e.g. protein (Li, K. et al. 2004). Hexamine (see Fig. 9B in Chapter 15 of this book) or the cheap methylolated nitroparaffins for example can functionally replace formaldehyde as hardener (Pizzi et al. 1997, Pichelin et al. 1999, Trosa & Pizzi 2001, Kim & Kim 2003, Kim et al. 2003, Pizzi 2006). In such way, panel boards satisfying the Japanese standard JIS A 5908 (2003) were obtained in laboratory and in industrial trials (Pichelin et al. 2006). In cases of poor bonding of difficult wood, mixtures with other types of (synthetic or bio-based) binders may overcome the problems (Pizzi 2000, 2006). Last but not least, different tannin sources and time of tannin addition in the process chain of MDF production (e.g. before or after defibration of wood chips) have been shown to influence of the characteristics of resulting boards (Dix & Schneider 2006).

Lignins

Lignin is a large, complex hydrophobic polymer build from different monolignol units, i.e. coniferyl alcohol, synapyl alcohol and *para*-coumaryl alcohol (Boerjan et al. 2003; see Fig. 2 in Chapter 7 and Fig. 3 in Chapter 21 of this book). After cellulose, lignin is the second most important component of plant biomass with an estimated 300 billion total tons in the biosphere on the earth and an annually re-synthesis of about 20 billion tons (Glasser & Kelly 1987). Worldwide, more than 50 million tons of lignin accumulate annually as by-product of pulp production (lignosulfonate from sulfite pulping processes and kraft lignin from sulfate pulping processes). These technical lignins are a yet sparsely used mass-product. About 10% of the technical lignins are exploited industrially whilst the rest is combusted or not utilised at all (Kharazipour et al. 1991, Northey 1992, Gargulak & Lebo 2000, Lora & Glasser 2002, Chakar & Ragauskas 2004, Gosselink et al. 2004a). With the invention of the “Pedersen-process” in the late 1960s, technical lignins have found for the first time on semi-industrial scale usage as binders in wood composite production (Pedersen & Rasmussen 1966, Roffael & Dix 1991).

Fibres obtained from a steam explosion process of wood chips display a highly lignin-rich, reactive surface structure that allows fibre adhesion in the production of binder-free hardboards (see also Chapters 15 and 18 of this book). The reactivity of the fibres is obtained by an accumulation of lignin from the inner cell wall layers on the surface and, most importantly, by a partial depolymerisation of fibre lignin via cleavage of alkyl-aryl (β -O-4) ether bonds between different monolignol units. This increases the phenolic hydroxyl content and produces relatively stable (phenoxy) radicals. During panel board pressing, the phenoxy radicals mediate polymerisation reactions leading to the adhesion of fibres (Felby et al. 1997b, Kharazipour et al. 1997, Anglès et al. 1999, Widsten 2002; further reading in

Chapter 18 of this book). The auto-adhesion of the fibre lignin can be further improved by increasing the temperature and by chemical treatment during the steam explosion processes. Enzymatic activation of the surface lignin, treatment of the fibres with Fenton's reagent ($\text{H}_2\text{O}_2/\text{FeSO}_4$; see Chapter 17 of this book) without or in presence of chelators such as 2,3-dihydroxybenzoic acid (DHBA), and irradiation with γ -radiation are further methods shown to increase auto-adhesion (Haars et al. 1989, Hüttermann et al. 1989, 2001, Kharazipour et al. 1991, 1997, Supe et al. 1993, Felby et al. 1997a,b, 1998, 2002, 2004, Widsten 2002, Widsten et al. 2002a,b,c, 2003a,b,c, 2004, Mai et al. 2004, Yelle et al. 2004; see Chapter 18 of this book). High-frequency ultrasound was recently also positively tested for increasing reactive groups of wood fibres (Gadhe et al. 2006). As the lignins on fibre surfaces, technical lignins are not as reactive and are therefore normally not applied as pure binder but as extenders for improving board properties, particularly the swelling properties. Chemical, electrochemical or enzymatic treatments prior to application as a binder can overcome at least partially the reaction problems (Nimz et al. 1972, 1976, Medvedev et al. 1985, Glasser 1989, Kuo et al. 1991, Stephanou & Pizzi 1993a,b, Chen & Wu 1994, Kharazipour et al. 1998, Hüttermann et al. 2000, 2001, Krumbiegel 2002, Feraud et al. 2006). Costs of such pretreatments and type and profitability of the wood composite products will however limit their applications in industry. Methylolating lignin prior is possible one of the economical most feasible pretreatments (Stephanou & Pizzi 1993a,b). Westin et al. (2001) reported that phenol-bonded MDF boards had an improved dimensional stability when kraft lignin was added but fixing lignin with a metal salt solution was required.

To improve reactivity it is also possible to add technical lignin to wood chips prior to defibration (Westin et al. 2001, Velazquez et al. 2003). In hardboards, addition of 3-8% kraft lignin made a post-heat-treatment unnecessary for reduction of swelling, regardless of when lignin was added prior or after fibre production (Westin et al. 2001). Hardboards made from the grass *Miscanthus sinensis* had a good quality when kraft lignin was added prior to defibration by steam explosion. Addition of lignin to the fibre pulp may however lead to bubble formation during high temperature pressing (Velazquez et al. 2003). In another study, water stability (thickness swelling and water absorption), internal bond, and mechanical properties were reported to be improved in panel boards made from fibres of softwood residues defibrated in presence of extra lignin (Anglès et al. 2001).

Its chemical properties and their structural similarity to PF resins make technical lignins attractive additives to mixed conventional adhesives (for detailed descriptions of conventional resins see Chapter 15 of this book), particularly in replacing phenol in phenol-formaldehyde binders. Formaldehyde from the PF resins is thought to act in such blends as a cross-linker between the reactive sites of the technical lignins and the synthetic phenol binder (Kuo et al. 1991, Vázquez et al. 1999, El Mansouri & Salvadó 2006, 2007). Many studies on replacing parts

of conventional PF- and also UF binders have been published and reviewed in the literature (Roffael & Rauch 1971, Shen 1974, Nimz 1983, Ayla & Nimz 1984, Abe 1987, Lewis & Lantzky 1989, Shiraishi 1989, Roffael & Dix 1991, Danielson & Simonson 1998a,b, Lisperguer & Solis 1999, Lisperguer et al. 2000, Feldman 2002, Turunen et al. 2002, Pizzi 2006, El Mansouri et al. 2007, Tejado et al. 2007a,b; see also Chapter 18 of this book). Replacing up to 50% of all phenol can give lignin-phenol-formaldehyde adhesives (LPFs) of equal or often also better bond strength but, for some lignin types, the thermo-stability decreases and pressing times may increase (Kazayawoko et al. 1992, Sellers et al. 1994, Zhao et al. 1994, Olivares et al. 1995, Vázquez et al. 1997, Danielson & Simonson 1998a,b, Wu & Zhan 2001, Cetin & Özmen 2002, Gosselink et al. 2004b, Khan et al. 2004a,b, Khan & Ashraf 2005). In view of economics, replacement of phenol by lignin in black liquor from kraft pulping is reported to reduce the cost of adhesives for bamboo plywood in China by a factor of 28.7% and, by national criteria, the produced bamboo plywood had better qualities (Wang, Y.Y. et al. 2006).

Technical lignins do not behave all in the same way. Kraft lignins and also soda-anthraquinone lignins appear to have better reaction properties than ligno-sulfonate, organosolv and ethanol process lignins (El Mansouri & Salvadó 2006). A novel sulfur-free lignin with the tradename NovaFiber behaves comparably to kraft lignin in terms of adhesive properties but it is easier to mix into glue matrixes and, moreover, said to be less smelling (Gosselink et al. 2004b). Combinations of kraft lignin with polyaminoamide-epichlorohydrin (PAE) and polyethylenimine (PEI) resin as cross-linking agents target at replacing formaldehyde-containing glues. Investigation on the curing chemistry of the new lignin-PEI adhesive revealed that phenolic hydroxyl-groups were oxidised to form quinones that further reacted with PEI (Li & Geng 2004, Geng & Li 2006, Liu & Li 2006).

Other bio-based adhesives

Unsaturated vegetable oils have in few cases (**linseed oil**) also been considered as adhesive. Resins on unsaturated oil basis employ epoxidation of the C-double bonds and cross-linking with a cyclic polycarboxylic acid anhydride. High prices and unsuitable long pressing times make them less attractive for implication in panel wood production (Pizzi 2006). There might be however some niche applications for oil-based resins.

Phenolic-rich softwood bark **pyrolysis oil (PO)** has been tested for OSB production of exterior grade and up to 35% of PF resin may be replaced by the oil (Chan et al. 2002). Application of formaldehyde-based resins mixed in parts with PO may help in protection of wood from fungal penetration (Mourant et al. 2007). Mixtures of isocyanates with POs in amounts as high as 40% give acceptable interior grade particle boards. Presence of POs in the binder strongly reduces metal adhesion of boards (Gagnon et al. 2004), reducing a special problem

occurring when using isocyanates (see Chapter 15 of this book). Replacement of synthetic phenol-formaldehyde resins by phenolics-rich, condensable pyrolysis tars obtained from carbonisation from softwoods and hardwoods has also been suggested (Ku & Mun 2006).

Renewable lignocellulosic material such as wood can be subjected to liquifaction in the presence of phenols (e.g. resorcinol) and an acidic or alkaline catalyst (Lin et al. 1994, 1995, Maldas & Shiraishi 1997a,b; see above). Resulting **liquified wood** with non-reacted phenols has adhesive properties (Lin et al. 1995, Maldas & Shiraishi 1997b) and is used in the design of various new types of binders (Kobayashi et al. 2000, Lee et al. 2000, Lee 2003, Wei et al. 2004, 2005, Xie & Chen 2005, Gosh & Sain 2006, Kishi et al. 2006, Sain et al. 2006, 2007).

Conclusions

In times of ecological and economical concerns on the usage of crude oils as raw material for wood adhesives, natural binders found their place back in wood composite production. Pure bio-based adhesives might be applied or combinations of bio-based adhesives with conventional synthetic resins or chemical replacements of these or compounds in these. Applications will depend on the special technical properties of the natural binders in glue preparation and during gluing and pressing as well as on the properties of the boards obtained after pressing. It is to be expected that in the future binder recipes and also wood composite production conditions will be further optimised to overcome any problems such as in binding strength and water resistance. Different applications of wood composites require different standards. Some more expensive and technically more complex binders might be designed and used just for niche applications. From the multitude of bio-based binders presented in the literature, not all will likely be implemented. If not for niche applications, only those bio-adhesives that fulfil various criteria (from abundant annually renewable resources, inexpensive, non-toxic before and during and after use, easy in handling, of best gluing properties and stabilities, water-resistance after gluing) will become successful. Of the different types of applied or potential natural binders, tannins and lignins are given special attention due to their structural similarity to the phenols in conventional synthetic resins. Value-added usage of technical lignins will furthermore solve for the pulp and paper industry a disposal problem of a little used by-product. Starch and certain plant proteins are easily available in larger amounts at cheap prices to volunteer in binder formulations appropriate for mass production of wood composite. Usually, bio-based binders are not pure solutions of one type of polymer. For example, the starch-rich potato-pulp contains also pectin and cellulose and large parts in wheat protein concentrates are indeed carbohydrates. Interactions between the different types of polymers and compounds contribute to the adhesive forces of the glues.

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17. Enzymes in Wood Degradation

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Introduction

Agricultural and forestry biomass provide the main source of renewable energy on earth and build the main sink for binding atmospheric CO₂ (see also Chapters 5 and 6 of this book). The major constituent of most living plants is lignocellulose (Watson et al. 2000), consisting from 90 to 98% of cellulose (40-50%), hemicellulose (27-31%), and lignin (20-30%) in the dry biomass (Eriksson et al. 1990, Morrell & Gartner 1998). Lignocellulose accounts for approximately 50% of the biomass in the world; its estimated annual production is 10-50 billion tons (Goldstein 1981, Lutzen et al. 1983). However, carbon cycling in nature would not be possible without effective degradation of lignocellulose. Degradation of the compo-

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nents of plant biomass - essentially polymeric compounds - also allows a transfer of the solar energy stored from photosynthesis to other organisms.

Wood biomass, as the most abundant lignocellulosic material, is mainly degraded by filamentous fungi that have specialised in using this substrate for their saprophytic, parasitic, or symbiotic life styles. Wood decaying fungi are commonly divided into three groups: i. white-rot fungi (mainly basidiomycetes) degrading extensive amounts of lignin present in the plant cell walls and causing whitening of the substrate, ii. brown-rot fungi (basidiomycetes) characterised by extensive degradation of cellulose and hemicellulose with limited ability to degrade lignin, and iii. soft-rot fungi (mostly ascomycetes and deuteromycetes) attacking wood with a high moisture content and causing cavities within the plant cell wall. Furthermore, white-rot fungi are further divided into two types, i.e. selective white-rot fungi degrading preferentially lignin and hemicellulose at the early stage of decay and simultaneous white-rot fungi breaking down lignin, cellulose, and hemicellulose at approximately the same rates (Eriksson et al. 1990, Schwarze et al. 2000).

The biodegradation of insoluble polymeric fractions of lignocellulose by fungi is achieved by extracellular enzymes and oxidative radical processes initiated by the organisms. The conversion of the isolated polysaccharide compounds cellulose and hemicellulose by various hydrolases is considered a rather simple process. However, when these polysaccharides occur in complexes with lignin in a way typical for woody plants, they are resistant against simple hydrolytic breakdown (Leonowicz et al. 1999a, 2001). Compared to the polysaccharides, lignin is structurally much more complex and made up primarily of phenyl propane units (see Fig. 7 in Chapter 2 and Fig. 3 in Chapter 21 of this book). The carbon-carbon and ether bonds joining the subunits together have to be cleaved via an oxidative mechanism. In order to attack this structurally rather randomly arranged heteropolymer, lignin degrading fungi have evolved complex but also unspecific enzyme systems (Breen & Singleton 1999).

Overview of enzymes involved in wood degradation

According to their role in the wood degradation process, enzymes can be divided into three groups (Table 1). The first group comprises oxidative enzymes directly attacking lignin structures and is responsible for oxidation of aromatic units and depolymerisation. The second group contains hydrolytic enzymes attacking cellulose and hemicellulose, degrading these polymers to small units and finally to monosaccharides. Other oxidative enzymes such as superoxide dismutase and glyoxalate oxidase build up the third group of enzymes that do not target the wood directly but cooperate with enzymes from the first group. These enzymes are responsible e.g. for production of hydrogen peroxide while oxidising glucose from hydrolytic degradation of cellulose or for reduction of quinones and radicals. According to the models proposed by Ander & Marzullo (1997) and Leonowicz et

al. (1999a), they play a key role in combining the different chains of metabolic reactions occurring during lignocellulose transformation.

Oxidative enzymes

Lignin degradation by white-rot fungi is performed by a group of oxidative enzymes that are extracellular and act in a consortium that may include also low molecular weight metabolites, e.g. aromatic compounds, organic acids, or peptides (de Jong et al. 1994, Goodell et al. 1997, Leonowicz et al. 1999a, 2001, Martínez et al. 2005). Physiological conditions for lignin degradation and patterns of involved enzymes may vary substantially among different species (compare e.g. Leonowicz et al. 2001 and Martínez et al. 2004). The most common enzymes for lignin degradation are described below.

Laccase (EC 1.10.3.2)

Laccases (synonyms benzendioloxygen oxidoreductase, urishiol oxidase, diphenol oxidase, *p*-diphenol:dioxygen oxidoreductase) belong to the family of multi-copper oxidases which among others include ascorbate oxidases, ceruloplasmin, and ferroxidases (Hoegger et al. 2006). Laccases catalyse the oxidation of various aromatic compounds such as mono-, di-, and polyphenols, aromatic amines, and methoxyphenols with a concomitant reduction of molecular oxygen to water by four one-electron transfers (Fig. 1). These enzymes were found in almost all wood-rotting fungi analysed so far (Heinzkill & Messner 1997, Baldrian 2006,

Table 1 Enzymes involved in the degradation of wood (adopted and modified from Leonowicz et al. 1999a)

Enzyme	Reaction
Oxidative enzymes - Lignin degrading enzymes	
Laccase (EC 1.10.3.2)	Oxidation of aromatic compounds such as mono-, di- and polyphenols, aromatic amines, and methoxy-phenols
Lignin peroxidase (EC 1.11.1.14)	Oxidation of alkyl side chains, C-C cleavage in side chain of lignin, cleavage of aromatic ring, oxidation of benzyl alcohols to aldehydes
Mn-dependent peroxidase (EC 1.11.1.13)	Various oxidations of aromatic and non-aromatic compounds
Mn-independent peroxidase (EC 1.11.1.16)	Various oxidations of aromatic and non-aromatic compounds
Protocatechuate 3,4-dioxygenase (EC 1.13.11.3)	$3,4\text{-Dihydroxybenzoate} + \text{O}_2 = 3\text{-carboxy-cis,cis-muconate}$
Catechol oxidase (EC 1.10.3.1)	$\text{Catechol} + \text{O}_2 = 1,2\text{-benzoquinone}$
Catechol 1,2-dioxygenase (EC 1.13.11.1)	$\text{Catechol} + \text{O}_2 = \text{cis,cis-muconate}$

Table 1 continued

Enzyme	Reaction
Hydrolytic enzymes - Cellulose degrading enzymes	
Endo-1,4- β -D-glucanase (EC 3.2.1.4)	Endohydrolysis of 1,4- β -D-glucosidic linkages
Exo-1,4- β -D-glucanases (EC 3.2.1.91, EC 3.2.1.4)	Hydrolysis of 1,4- β -D-glucosidic linkages releasing cellobiose or glucose
β -D-Glucosidase (EC 3.2.1.21)	Hydrolysis of terminal nonreducing β -D-glucose residues with release of β -D-glucose
Hydrolytic enzymes - Enzymes acting on hemicellulose from hardwood (xylan)	
Endo-1,4- β -xylanase (EC 3.2.1.8)	Endohydrolysis of 1,4- β -D-xylosidic linkages in xylans
1,4- β -D-Xylosidase (EC 3.2.1.37)	Hydrolysis of 1,4-D-xylans to remove successive D-xylose residues
α -L-Arabinofuranosidase (EC 3.2.1.55)	Hydrolysis of terminal nonreducing α -L-arabinofuranoside residues in α -L-arabinosides
Acetyl esterase (EC 3.1.1.6)	Acetic ester + H ₂ O = alcohol + acetate
α -D-Glucuronidase (EC 3.2.1.139)	α -D-glucuronoside + H ₂ O = an alcohol + D-glucuronate
Hydrolytic enzymes - Enzymes acting on hemicellulose from softwood (mannan)	
Endo-1,4- β -D-mannosidase (EC 3.2.1.78)	Random hydrolysis of 1,4- β -D-mannosidic linkages in mannans
Exo-1,4- β -D-mannosidase (EC 3.2.1.25)	Hydrolysis of terminal, nonreducing β -D-mannose residues in β -D-mannosides
Exo-1,3- β -D-galactosidase (EC 3.2.1.145)	Hydrolysis of terminal, non-reducing β -D-galactose residues in 1,3- β -D-galactans
α -D-Galactosidase (EC 3.2.1.22)	Hydrolysis of terminal α -D-galactose residues in α -D-galactosides
Enzymes supporting the lignocellulose degradation	
Glyoxylate oxidase (EC 1.2.3.5)	Glyoxylate + H ₂ O + O ₂ = oxalate + H ₂ O ₂
Glucose oxidase (EC 1.1.3.4)	β -D-Glucose + O ₂ = D-glucono-1,5-lactone + H ₂ O ₂
Aryl alcohol oxidase (EC 1.1.3.7)	An aromatic primary alcohol + O ₂ = aromatic aldehyde + H ₂ O ₂
Pyranose oxidase (EC 1.1.3.10)	D-Glucose + O ₂ = 2-dehydro-D-glucose + H ₂ O ₂
Cellobiose dehydrogenase (EC 1.1.99.18) - processed as cellobiose:quinone oxidoreductase	Cellobiose + acceptor = cellobiono-1,5-lactone + reduced acceptor
Carboxylesterase, e.g. feruloyl esterase (EC 3.1.1.73), coumaryl esterase (no EC number)	Ferulic acid ester + H ₂ O = alcohol + ferulic acid Coumaryl acid ester + H ₂ O = alcohol + coumaryl acid

Hoegger et al. 2006). Although one of the strongest lignin degrading species, *Phanerochaete chrysosporium*, does not produce a typical laccase (Srinivasan et al. 1995, Larrondo et al. 2003b, Vanden Wymelenberg et al. 2006a,b), it is evident from studies of other species that laccases can play an important role in the lignin de-

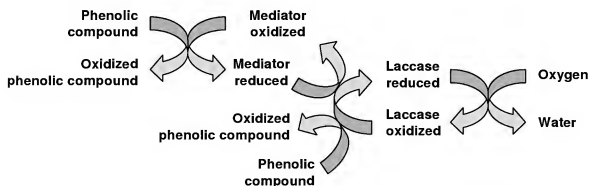


Fig. 1 Oxidation of phenolic compounds by laccase and laccase-mediator systems (adopted from d'Acunzo et al. 2004)

gradation process (Leonowicz et al. 2001, Baldrian 2006). In the white-rot fungus *Pycnoporus cinnabarinus*, which secretes neither lignin nor manganese peroxidase, laccase is essential for lignin degradation (Eggert et al. 1997, Bermek et al. 1998). Although laccase of *P. cinnabarinus* acts much more efficiently on phenolic lignin substructures, the enzyme also appears to help in degradation of non-phenolic lignin substructures, possibly via some kind of mediator reaction (Geng & Li 2002). A **mediator** is a small organic molecule with a high redox-potential that, once oxidised by a laccase, can oxidise other phenolic or non-phenolic molecules by transfer of electrons (Bourbonnais et al. 1997, Kawai et al. 1999, 2006, Li et al. 1999, Morozova et al. 2007). In such a way, the rate of oxidation of aromatic laccase substrates can increase and, in addition, compounds not being substrates of the enzymes might be oxidised (Fig. 1; see below and also Chapter 18 of this book). Natural mediators for laccases are phenol, aniline, 4-hydroxybenzoic acid, N-hydroxyphthalimides, and 4-hydroxybenzyl alcohol (Johannes & Majcherczyk 2000, Annunziatini et al. 2005).

Lignin peroxidase (LiP; EC 1.11.1.14)

This type of enzyme (synonym ligninase) was first discovered in nitrogen-limited cultures of *P. chrysosporium* (Glenn et al. 1983, Tien & Kirk 1983) and later also found in other white-rots, e.g. *Phlebia radiata* and *Trametes versicolor* (Hatakka et al. 1987, Jonsson et al. 1987, Hatakka 1994, Pointing et al. 2005). However, probably only 40% of white-rot fungi possess this enzyme. *P. cinnabarinus*, *Cyathus stercoreus*, *Dichomitus squalens*, and *Ceriporiopsis subvermispura* for example degrade lignin without any detectable LiP activity (Péridé & Gold 1991, Eggert et al. 1996b, Jensen et al. 1996, Sethuraman et al. 1999). LiP contains a heme in the active site (Banci et al. 1991, Choinowski et al. 1999) and requires hydrogen peroxide to function (Wariishi et al. 1990, Ferapontova et al. 2006). The catalytic cycle of LiP is similar to other peroxidases. The reaction of the native iron-containing enzyme (Fe-LiP) with H_2O_2 yields the redox state LiP-compound I (LiPI), a complex of a

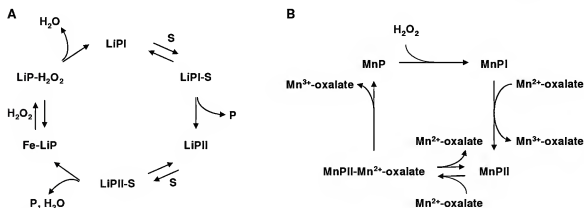


Fig. 2 A. The catalytic cycle of oxidation of phenolic substrates (S) by lignin peroxidases; P = product (after Ward et al. 2003) and B. the mechanism of manganese peroxidase catalysed formation of Mn^{3+} -oxalate complexes (after Zapanta & Tien 1997)

high valence oxo-iron (localised in the heme porphyrin structure) and a radical porphyrin cation. A one-electron oxidation of a reducing substrate by LiP yields a cation radical and a one-electron-oxidised enzyme intermediate (redox state LiP-compound II; LiPII). A subsequent one-electron oxidation of a second substrate molecule by LiPII results in the formation of another radical cation and the release of a molecule water whilst the enzyme reverts into its original Fe-form (Ward et al. 2003; Fig. 2A). LiP has been characterised as a veratryl alcohol oxidising enzyme (Khindaria et al. 1995). Veratryl alcohol as a fungal metabolite (Khindaria et al. 1995, Mester et al. 1997) is transformed by LiP into a cation-centered radical (de Jong et al. 1994) which complexes to the enzyme to possibly function as a redox mediator in transformation reactions of other compounds (Koduri & Tien 1994, Khindaria et al. 1996, Baciocchi et al. 2002). LiP thus is a relatively unspecific enzyme catalysing a variety of reactions with hydrogen peroxide as the electron acceptor. It oxidises e.g. alkyl sides of aromatic compounds, aromatic alcohols, and benzylic methylene groups. LiP is able to oxidise highly recalcitrant aromatic structures with a redox potential of about 1.5 V and is also capable to initiate cleavage of an aromatic ring by one-electron oxidation and subsequent radical reactions. It also cleaves C-C bonds in side chains of lignin subunits (Kersten et al. 1985; Fig. 3). In contrast to earlier assumptions (Kirk & Farrell 1987), oxidations performed by LiP do not necessarily require veratryl alcohol as a mediator compound but this metabolite stimulates and protects the enzyme (Harvey et al. 1986, Huang et al. 2003).

Manganese peroxidase (MnP; EC 1.11.1.13)

This type of enzyme was discovered one year after LiP, also from N-depleted cultures of *P. chrysosporium* (Kuwahara et al. 1984) and belongs as LiP to the fungal class II peroxidases (Larrondo et al. 2005). MnP, similarly to LiP, requires H_2O_2 as

*image
not
available*

and substituted phenols. In addition, they are able to oxidise Mn^{2+} to Mn^{3+} , thus combining partially functions of MnP and of LiP. Enzymes of this type are called manganese-independent or versatile peroxidases (VPs). They require hydrogen peroxide as a co-substrate but do not depend on mediators (Martínez et al. 2005, Pogni et al. 2005).

Other peroxidases

Horseradish peroxidase (EC 1.11.1.7) like lignin peroxidase is able to attack non-phenolic β -O-4 lignin models in the presence of hydrogen and HBT (1-hydroxybenzotriazole) as a mediator, thereby producing various ring-cleavage products as well as a C_{α} - C_{β} cleavage product (Kawai et al. 2006, compare Fig. 3). Heme-containing, H_2O_2 -depending enzymes with properties similar to horseradish and other plant peroxidases have been described from the white-rots *Inonotus radiatus* and related species, *Pholiota mutabilis* and relatives, and *T. versicolor* where they are believed to contribute to lignin degradation. Such plant-like manganese-independent peroxidases from *T. versicolor* can polymerise and depolymerise lignin similar to LiP and MnP but do not react with veratryl alcohol (Lobaczewski et al. 1982, Lobaczewski 1990, Stoychev et al. 1998). Horseradish peroxidase similar enzymes are also present in saprophytic fungi, e.g. **peroxidase Cip** in *Coprinopsis cinerea* (Heinzkill et al. 1998, Morita et al. 1988). This class II fungal peroxidase is used as a peroxidase model and thus being one of the best analysed peroxidases (for details on structure, catalytic properties and reaction mechanism, etc. see Ryu et al. 1995, Abelskov et al. 1997, Ciaccio et al. 2003, Houborg et al. 2003a,b, Schiodt et al. 2007; see above). However, Cip acts on various phenolic compounds, but not on lignin molecules (Petersen et al. 1994, Aitken & Heck 1998, Ikeda et al. 1988, Masuda et al. 2001).

Dioxygenases: protocatechuate 3,4-dioxygenase (EC 1.13.11.3), catechol oxidase (synonyms tyrosinase, phenolase; EC 1.10.3.1), catechol 1,2-dioxygenase (EC 1.13.11.1), and hydroxychinol 1,2-dioxygenase (EC 1.13.11.37)

Dioxygenases are widely distributed in micro-organisms and were also isolated from wood-degrading fungi (Lindeberg & Holm 1952, Matsubara & Iwasaki 1972, Hofrichter et al. 1994, Ratcliffe et al. 1994, Rieble et al. 1994). Dioxygenases are intracellular enzymes with two copper atoms in the active site capable of oxidising phenolic compounds (Balogh-Hergovich & Speier 1994). Unlike laccases, these enzymes hydroxylate phenolic compounds, oxidise diphenols to quinones and cleave the aromatic rings (Vaillancourt et al. 2006; compare Table 1). Hydroxychinol (1,2,4-trihydroxybenzene) 1,2-dioxygenase for example catalyses an intradiol cleavage of the aromatic ring in vanillate, to produce maleylacetate, as a key step in the degradation pathway of lignin (Rieble et al. 1994). Fungal protocatechuate 3,4-

dioxygenase was shown to cleave the aromatic moiety of lignosulphonate (Wojtas-Wasilewska et al. 1983, 1988). This latter enzyme catalyses the first step in the pathway of protocatechuate (derived from phenolic compounds including *p*-cresol, 4-hydroxybenzoate, and numerous lignin monomers) to β -ketoadipate. Alternatively, β -ketoadipate is obtained from catechol (derived from various aromatic hydrocarbons, amino aromatics, and lignin monomers) with catechol 1,2-dioxygenase performing the first step in the conversion (Harwood & Parales 1996). Tyrosinase activity has occasionally been reported in white-rots (Nsolomo et al. 2000, Tomšovský & Homolka 2004, Koukol et al. 2006). However, commercial mushroom tyrosinase caused in laboratory trials only negligible effects on milled wood (Grönqvist et al. 2005).

Other oxidoreductases

Several other enzymes secreted by fungi perform oxidative reactions and are believed to participate in lignin degradation processes (see also Table 1). **Glucose oxidase** (β -D-glucose:oxygen 1-oxidoreductase; EC 1.1.3.4), **pyranose oxidase** (pyranose:oxygen 2-oxidoreductase, glucose 2-oxidase; EC 1.1.3.10), and **glyoxalate oxidase** (glyoxylate:oxygen oxidoreductase; EC 1.2.3.5) are FAD (flavin adenine dinucleotide)-dependent enzymes capable to oxidise sugars by reducing oxygen to hydrogen peroxide. These reactions seem to be the most important sources of H_2O_2 which is required as co-substrate for peroxidases (see above) as well as an oxidant in Fenton-like reactions ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^-$; $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^\bullet + H^+$) giving rise to reactive oxygen species capable to penetrate wooden cell walls and initiate their decay (Hammel et al. 2002, Henry 2003).

Another source of hydrogen peroxide is the FAD-dependent enzyme **aryl-alcohol oxidase** (EC 1.1.3.7). This type of enzyme is capable to oxidise aromatic alcohols to the corresponding aldehydes by concomitantly reducing oxygen to H_2O_2 and is found in many wood-degrading fungi (Muheim et al. 1990, Guillen & Evans 1994, Asada et al. 1995, Barrasa et al. 1998, Varela et al. 2000, Kim et al. 2001, Ferreira et al. 2005). Furthermore, a new group of small **Fe^{2+} -containing glycoproteins** has recently been reported to catalyse redox reactions between an electron donor, such as NADH, and O_2 in order to produce H_2O_2 and to reduce H_2O_2 to OH^\bullet (Tanaka et al. 1996, 1999, 2007, Enoki et al. 2003). Another novel small peptide from *P. chrysosporium* (Pc factor) has phenol oxidase activity and reduces Fe^{3+} to Fe^{2+} but appears not to form OH^\bullet (Hu et al. 2006), showing that the list of fungal oxidoreductases with potential functions in lignocellulose degradation is likely not completed.

Quinones, produced by oxidation of lignin for example by actions of laccase (Guillen et al. 2000), peroxidases (Gomez-Toribio et al. 2001), or by photodegradation of wood (Mitsui & Tsuchikawa 2005) may undergo a back reduction to the

original phenols (see above). During the reduction of quinones, the enzyme **cellobiose dehydrogenase (CDH; EC 1.1.99.18)** oxidises cellobiose to cellobionolactone (Zamocky et al. 2006). A similar activity is assigned to **cellobiose:quinone oxidoreductases (CBQ)**, for a long time thought to be a separate enzyme. Later CBQ was proved to be a proteolytic product of CDH (Eriksson et al. 1993, Ander & Marzullo 1997, Henriksson et al. 2000), the only known extracellular flavocytochrome that is found in a variety of white-rot, brown-rot and soft-rot wood-degrading fungi but also in soil fungi and phytopathogens (Zamocky et al. 2006).

Moreover, **glucose oxidase (EC 1.1.3.4)** may support degradation of lignin through reduction of quinoid compounds that hence will not undergo any polymerisation actions and, in the course of this, overall lignin depolymerisation will be enhanced (Leonowicz et al. 1999b, 2001).

Hydrolytic enzymes: Enzymes degrading cellulose

Degradation of lignin by wood rotting fungi is understood as the removal of a physical barrier to reach the ultimate energy source, polysaccharides (Eriksson et al. 1980). Although fungi can at least partially catabolise lignin degradation products, they can not use them as the only energy source. Therefore, wood-degrading fungi possess complex enzymatic mechanisms allowing them to effectively degrade the sugar polymers cellulose and hemicellulose. Cellulose is the major component of wood and comprises 40 to 45% of the wood biomass (Willför et al. 2005). It is a linear 1,4- β -D-glucopyranose polymer that in woody species accounts for about 14,000 glucose units in one polymer molecule (see Fig. 2 in Chapter 21 of this book). Glucan chains of cellulose have a twofold screw axis of symmetry stabilised by intra- and intermolecular hydrogen bonds. Within the lignocellulose structure, cellulose is present in the so called amorphous and the crystalline form (Andersson et al. 2003, 2004, Thygesen et al. 2005). The crystalline form possesses a higher resistance towards enzymatic degradation compared to the amorphous cellulose (Sethuraman et al. 1998). The latter is degraded by numerous micro-organisms in nature (Schwarz 2001, Lynd et al. 2002) whereas the crystalline form is degraded only by a limited group of organisms, mostly fungi (particularly brown-rots) able to produce a cellulolytic enzyme system consisting of endo-1,4- β -glucanase, exo-1,4- β -glucanase, and 1,4- β -glucosidase (see e.g. Cohen et al. 2005, Yoon & Kim 2005, Boonstra et al. 2006, Irbe et al. 2006; see Table 1).

Endo-1,4- β -D-glucanase (cellulase; EC 3.2.1.4) hydrolyses cellulose chains in a random way producing shorter chains with non-reducing ends. This process delivers substrates for the **exo-1,4- β -D-glucanase (cellulose 1,4- β -cellobiosidase; EC 3.2.1.91)** which commonly removes dimers (cellobiose) from the non-reducing end of poly- and oligosaccharides. Final hydrolysis of cellobiose to two

glucose units is performed by **1,4- β -D-glucosidase (cellobiase; EC 3.2.1.21)**. However, this hydrolytic enzymatic principle of cellulose degradation is rather simplified and other, e.g. oxidative, processes are also important for fungal degradation of cellulose. The cellulose degradation processes are strongly enhanced by activities of CDH or cellobiose oxidase (Bao & Renganathan 1992). Several functions of CDH in the cellulose degradation have been proposed: i. CDH oxidises free ends created by endocellulases and prevents re-condensation of the cellulose chain (Ayers et al. 1978), ii. CDH removes cellobiose and thus prevents inhibition of cellulases (Ayers et al. 1978), and iii. CDH reduces Fe^{3+} to Fe^{2+} while producing H_2O_2 and thus supports formation of hydroxyl radicals in a Fenton-type reaction (see above) which depolymerises or modifies the cellulose (Kremer & Wood 1992, Kruså et al. 2005). In the degradation of cellulose by brown-rot fungi, the role of low molecular compounds, peptides, and metal ions seems to be more important. Since many of these latter fungi are known to lack the exoglucanases, oxidative processes involving non-enzymatic routes such as the Fenton-based free radical mechanism were postulated to be necessary for cellulose degradation (Goodell 2003).

Hydrolytic enzymes: Enzymes degrading hemicellulose

Early chemical analysis of wood components at the end of the nineteenth century revealed a sugar polymer extractable from wood fibre with diluted alkali. This polymer was believed to be a precursor of the insoluble cellulose and was named hemicellulose (Schulze 1891). Nowadays, this term covers a range of branched polysaccharides composed of different proportions of monosaccharides such as the hexoses D-mannose, D-glucose, and D-galactose, the pentoses D-xylose and L-arabinose, and the sugar acids (hexuronic acids) D-glucuronic acid and D-galacturonic acid (Fig. 4). Therefore, hemicellulose is not a defined compound but rather describes a class of polymers in plant cells. Single polymers are commonly named by the type of sugar building its main chain (backbone), e.g. mannans are made up mostly of mannose and glucuronoxylans are made up of D-glucuronic acid and D-xylose, respectively (Ebringerová et al. 2005).

The major **hardwood hemicellulose** consists mainly of β (1-4)-glycosidic linked D-xylose polymers, hence named **xylan**. Xylans present between 15-30% of the hardwood biomass. Their linear backbones contain in total about 150-200 xylose residues. On average, each tenth xylose is 1,2-linked with 4-O-methyl-glucuronoxylans and 60-70% of the xylose residues in the backbone are O-acetylated at positions 2 or 3 (Fig. 5). In addition to xylans, other types of hemicelluloses are found in hardwood but in lower amounts. Of these minor hemicelluloses, **glucomannan** represents still 2-5% of the hardwood biomass. Hardwood glucomannan is a linear polymer of varying ratios of β -D-mannose and β -D-glucose linked by 1,4-bonds (Timell 1964, Sjöström 1981, Saha 2003; Fig. 5).

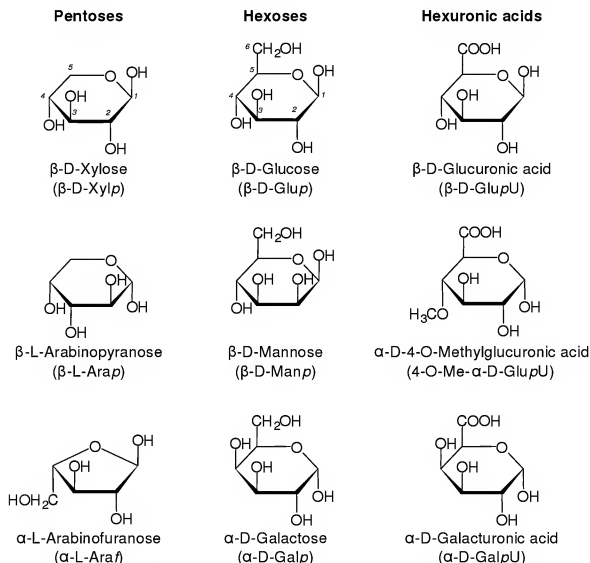


Fig. 4 Haworth projections of the structures of the main monosaccharides building up hemicelluloses in wood. For simplification of the figure, only the C positions in β -D-xylose and β -D-glucose are numbered

Due to its complexity and heterogeneity, the degradation of the xylans is performed by a complex mixture of hydrolytic enzymes catalysing the breakdown to simple structures and finally to monosaccharides. Accordingly, these enzymes have been named **xylanases**. Enzymes degrading the glucomannans are endo-1,4- β -D-glucanases acting also on celluloses (Ogawa et al. 2007; see above).

Many micro-organisms possess complex sets of enzymes enabling complete breakdown of hemicelluloses. The most important components of these enzyme systems for the degradation of the major hardwood xylans are **endo-1,4- β -D-xylanase** (EC 3.2.1.8), **1,4- β -D-xylosidase** (EC 3.2.1.37), and the accessory enzymes **α -glucuronidase** (EC 3.2.1.139) and **acetyl esterase** (EC 3.1.1.6) (Beg et

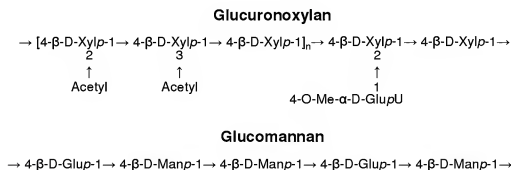


Fig. 5 Representative structures of the main hemicelluloses from hardwood (for abbreviations and chemical structures see Fig. 4)

al. 2001, de Vries & Visser 2001, Saha 2003, Polizeli et al. 2005; see Table 1 and below). Endo-1,4- β -xylanases attack the main chain of xylans to cleave the glycosidic bonds within the xylan structure (compare Fig. 5). The resulting oligomers can be further hydrolysed by this group of enzymes to tri-, di-, and monosaccharides. The enzymes can differ in their selectivity towards the xylan structure, composition, or branching. Therefore, most fungi secrete multiple forms of endoxylanases (Polizeli et al. 2005). Oligosaccharides resulting from xylan degradation by endoxylanases are effectively hydrolysed to xylose, the component monosaccharide, by 1,4- β -D-xylosidases (for fungal examples see De Almeida et al. 1995, Kiss & Kiss 2000, Katapodis et al. 2003, Katapodis 2006). Such enzymes are therefore necessary for the complete degradation and microbial utilisation of xylan (Sunna & Antranikian 1997, Saha 2003). Besides, the removal of oligosaccharides by xylosidases affects also endoxylanases by preventing the feedback inhibition exerted on these enzymes by their oligosaccharide products (Aro et al. 2005).

The composition of **hemicelluloses in softwood** with comparably low amounts of xylans is totally different from hardwood. Main softwood hemicelluloses are 70 to 130 units-long polymers with **galactoglucomannan** (also shortly referred to as glucomannans by the fact that only a low percentage of the polymer is made up by D-galactose) backbones (Fig. 6). Galactomannans form about 10-15% of softwood biomass whereas two other types of hemicelluloses, **arabinoglucuronoxylans** (also called arabinoxylans) and **arabinogalactans** (both shown in Fig. 6), count for 7-10% and 5-8% of softwood biomass, respectively (Timell 1964, Sjöström 1981, Saha 2003). In galactoglucomannans, galactosyl side chains attach to D-mannose and D-glucose residues and in addition up to half of the D-mannose residues might be acetylated at C-2 and C-3 positions (Fig. 6). Arabinoglucuronoxylan has a backbone purely made by β (1-4)-glycosidic linked D-xylose polymers. This backbone is partially substituted at C-2 by α -D-4-O-methylglucuronic acid residues and 1,3-linked with α -L-arabinofuranose. In the softwood xylans, arabinofuranosyl residues can be esterified with *p*-coumaric acids or ferulic

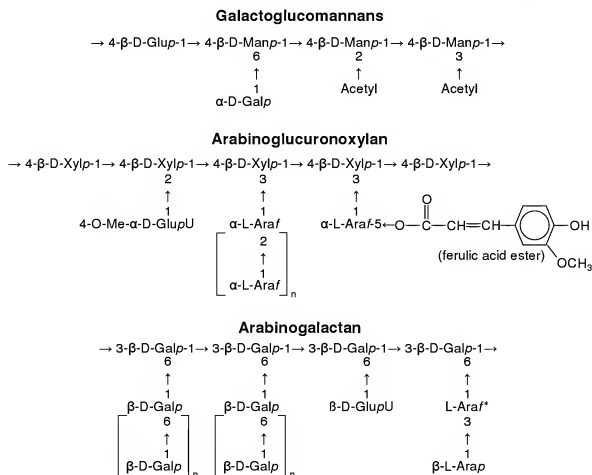


Fig. 6. Representative structures of the main hemicelluloses from softwood (for abbreviations and chemical structures see Fig. 4; * denotes that at this position either $\alpha\text{-L-Araf}$ or $\beta\text{-L-Araf}$ might be inserted)

acids (Fig. 6). In arabinogalactans, the $\beta(1-3)$ -glycosidic linked D-galactose backbone is heavily substituted at the C-6 position of the galactoses by mono- and oligosaccharide side chains composed of $\beta\text{-D-galactose}$, L-arabinofuranose, $\beta\text{-L-arabinopyranose}$, or $\beta\text{-D-glucuronic acid}$ (Fig. 6).

Although research on softwood hemicelluloses is limited, similar pathways as described above for degradation of the main hardwood xylans exist also for degradation of the different types of softwood hemicelluloses. These pathways involve analogous groups of hydrolytic enzymes, e.g. endomannanases, mannosidases, galactosidases, glucosidases, etc. (Pandey & Soccol 2000, Howard et al. 2003, Thygesen et al. 2003, Gubitz et al. 1996, Valášková & Baldrian 2006a,b, Gomez et al. 2007; Table 1). Enzymes acting on the main types of softwood hemicelluloses are **endo-1,4- $\beta\text{-D-mannanases}$ (EC 3.2.1.78)** and **exo-1,4- $\beta\text{-D-mannosidases}$ (EC 3.2.1.25)** in case of galactoglucomannan, **endo-1,4- $\beta\text{-D-xy-$**

lanases (EC 3.2.1.8) in case of arabinoglucuronoxylan, and **1,3- β -D-galactosidases (EC 3.2.1.145)** in case of arabinogalactan, respectively (see Table 1). Further to the above mentioned feedback inhibition, an effective degradation of the softwood xylans by xylanases can be hindered by the presence of side chains such as L-arabinose residues 1,3-linked to the arabinoglucuronoxylan backbone (Fig. 6). Hydrolysis of the terminal non-reducing α -L-arabinose in e.g. arabinoxylans or also arabinogalactans is performed by the accessory enzyme **α -L-arabinofuranosidase (EC 3.2.1.55)** (Saha 2000, 2003). Similarly, glucuronic acid residues linked to the xylan backbone (Fig. 6) are removed by **α -glucuronidase (EC 3.2.1.139)** (Pell et al. 2004). A high degree of hydroxyl groups of sugar residues in xylans are esterified by acetyl groups (see above) which also prevents enzymatic cleavage by hydrolytic enzymes (Kormelink & Voragen 1993, Wood & McCrae 1986, Cybinski et al. 1999, Silveira et al. 1999). During fungal degradation of wood, **acetyl esterase (EC 3.1.1.6)** removes O-acetyl groups from the positions C-2 and C-3 of β -D-xylopyranosyl residues in the xylan backbones (Halgasova et al. 1994, Tenkanen 1998, Cybinski et al. 1999, Chung et al. 2002, Ding et al. 2007). Esters of aromatic acids and arabinofuranose in arabinoglucuronoxylans (Fig. 6) are hydrolysed by **carboxylesterases, e.g. feruloyl esterases (EC 3.1.1.73)** and **coumaryl esterase** (Tenkanen 1998, Wong 2006). All these various types of hemicellulases might be produced by a fungus simultaneously to cellulases (Gubitz & Steiner 1995, Sachslehner et al. 1997, 1998, de Vries and Visser 2001, Howard et al. 2003, Polizeli et al. 2005; see Table 1).

Applications

Due to their high versatility, the fungal enzymatic systems for lignocellulose degradation have a huge potential for biotechnological applications (Viikari 2003; see also Chapters 18, 19, and 21 of this book). This versatility is demonstrated e.g. by the high diversity of compounds that can be degraded by the white-rotter *P. chrysosporium*: next to the biopolymers cellulose and lignin, an ever-growing list of chemicals covering synthetic polymers, chlorinated and polycyclic aromatic compounds, dyes, explosives, pesticides, and others are degraded mainly by extracellular enzymes normally involved in wood decay (Cameron et al. 2000). White-rot fungi and their enzymes therefore have a special standing in bioremediation of xenobiotics and other recalcitrant organic compounds in wastes of industrial origins that challenge the integrity of our environment (Gianfreda & Rao 2004, Tortella et al. 2005; Šušla & Svobodová 2006).

Of the various enzymes produced by the fungi to degrade wooden material, cellulases, xylanases, laccases, and peroxidases have found most attention for applications. Laccases and peroxidases for industrial applications tend to come from white-rot fungi (Ikehata et al. 2004; see Chapter 19 of this book). Enzymes degrading xylan and other components of hemicellulose were found not only in all

kind of wood-degrading fungi, but also in other fungi and bacteria (Eriksson et al. 1990). Xylanases are produced by mesophilic, thermophilic and even extremophilic organisms (Haki & Rakshit 2003, Techapun et al. 2003). Fungal xylanases from mesophilic species are relatively stable and possess temperature optima at 60–80°C and highest enzyme activities in the pH range of 4.5 to 6.5 and are therefore often choice for industrial applications (Polizeli et al. 2005). Whilst xylanases play a crucial role in wood degradation processes by wood rotting fungi (Eriksson & Goodell 1974, Majala et al. 1995, Liers et al. 2006, Valášková & Baldrian 2006a,b; see above), the most important sources for large scale industrial production of these enzymes are currently saprotrophic asco- and deuteromycetes such as *Aspergillus*, *Trichoderma*, and *Humicola* species. Likewise, species of these clades are also prominent producers of cellulases (Polizeli et al. 2005; Sukumaran et al. 2005; see Chapter 19 of this book). In addition, the spectrum of enzymes provided by fungi is further expanded by enzymes with cellulolytic and hemicellulolytic activities that are obtained from bacteria (for a selection of recent reviews on production and applications of the various enzymes see Sunna & Antranikian 1997, Cavaco-Paulo 1998, Bhat 2000, Pandey & Soccol 2000, Beg et al. 2001, Minussi et al. 2002, Galante & Formantici 2003, Saha 2000, 2003, Polizeli et al. 2005, Sukumaran et al. 2005, Couto & Herrera 2006, Husain 2006, Kilaru 2006, Šušla & Svobodová 2006; Schäfer et al. 2007, and also Chapter 19 of this book).

Cellulolytic enzymes

Cellulolytic enzymes are among the most important enzymes produced on industrial scale with best established applications (Galante & Formantici 2003, Schäfer et al. 2007). They are used in food industry, beer and wine production, upgrading of raw materials for animal feed, and in agriculture (Bhat 2000). In the textile industry, cellulolytic enzymes are successfully applied in producing the stone-washed look of denim, in deinking, in improving of textile appearance by removing fuzz fibres and pills, and in softening of garments (Cavaco-Paulo 1998).

In contrast, application of these enzymes in wood processing industries is rather limited in comparison to oxidative enzymes and xylanases (see below). However, special attention reached a subunit of many cellulases: the cellulose-binding domain (CBD). CBD commonly contains 30–180 amino acids and exists as a single, double, or triple domain within the protein (Hilden & Johansson 2004). It is a functionally independent subunit, essential for effectiveness of enzymatic hydrolysis and responsible for attaching of the enzyme to the surface of the cellulose fibre and thereby directing the catalytic domain of the protein towards the substrate. Treatment of cellulose pulps with recombinant CBD improves pulp drainability in filtration processes and mechanical properties of paper sheets (Pala et al. 2003). Fungal endoglucanases mixed with endoxylanase have been shown to help in deinking of mixed office waste paper (Marques et al. 2003) and the cellulo-

lytic enzymes have been reported in deinking to be as efficient as chemical treatments (Pala et al. 2004, 2006).

The biggest challenge in cellulase research at present is in the field of biofuel/bioethanol production. In 2006, total bioethanol production in the USA reached 18.9 billion litres (Gray 2007). Corn is used for ethanol production in the USA, and cane juice in Brazil being the other leading producer of biofuel (Solomon et al. 2007). Enzymatic saccharification of lignocellulosic feedstocks as a huge and cheap natural resource is an attractive alternative (Sheehan & Himmel 2001, Zaldivar et al. 2001, Sukumaran et al. 2005, Gray 2007, Kristensen et al. 2007, Ohgren et al. 2007, Solomon et al. 2007; see also Chapter 6 of this book). In the year 2001, costs for cellulase were estimated \$ 0.16 per l bioethanol obtained with currently available technology from cellulose. These costs for cellulases have to be reduced at least tenfold to be economical. Reaching this target requires a tenfold increase in enzyme-specific activities or enzyme production efficiency or combinations thereof (Sheehan & Himmel 2001, Zhang et al. 2006). Recombinant DNA technologies might help to improve production yields and enzyme properties (see below and Chapter 19 of this book).

Xylanases

Xylanases present another large product group on the enzyme market which are applied individually or in combination with other enzymes in industrial production processes (Schäfer et al. 2007). For example, they are used to reduce the viscosity and increase the digestibility of raw materials for upgrading animal fodder, in manufacture of bread, food, and drinks, or in the chemical industry for production of xylan derivatives (e.g. xylitol, sugars, and ethanol). However, the main area of application of xylanases is in fibre processing. Xylanases free of cellulases are important reagents in the paper and textile industry (Bajpai 1999, Beg et al. 2001, Polizeli et al. 2005).

Complete removal of lignin is an essential step in production of high quality cellulose pulp and paper. Chemical bleaching of pulp with chlorine dioxide, oxygen, or hydrogen peroxide performed to remove the lignin residues can be minimised by pre-treatment of the raw material with xylanases that removes about 80% of fibre associated lignin (Tolan & Popovici 2003, Polizeli et al. 2005). The enzymes are supposed to remove the xylan precipitated on the fibre surface and therefore to expose the lignin structures to chemical treatments (Viikari et al. 1994) or to remove alkali-resistant hemicellulose-lignin complexes by hydrolysis of the polysaccharide component (Buchert et al. 1993, Schönberg et al. 2001). Degradation of hemicellulose and the concomitantly facilitated removal of lignin from the cellulose fibres prevent the darkening of the products caused by lignin oxidation (Tolan & Popovici 2003).

Laccases

Laccases require oxygen as a co-substrate which is readily available for the enzyme as water-dissolved atmospheric gas (Yaropolov et al. 1994). The low costs associated with this electron acceptor in the oxidation reaction make laccases very interesting enzymes for various biotechnological applications. Most of the proposed uses for laccases are based on the ability to produce a free radical from a suitable substrate. The numerous secondary reactions of the radicals are responsible for the versatility of possible applications (Mayer & Staples 2002, Kilaru 2006). For example in the food and beverage industry, laccases can stabilise wine, musts, beer, or fruit juices through oxidation and polymerisation of polyphenolic substances. Such stabilisation can prevent phenol-dependent discolouration, unwanted changes in taste, or turbidity. Application of laccases can have beneficial effects on the flavour of foodstuff and, in baking, it improves dough and bread characteristics by strengthening the gluten structures (reviewed in Minussi et al. 2002). Applications for laccases have been proposed in health care products such as mouthwash, toothpaste, mints, and gums in order to prevent oral malodour (Brinch & Pedersen 2002), and in cosmetics for lightening the skin (Golz-Berner et al. 2004) or dyeing human keratin fibres (Josse 2000). In the leather industry, laccases may be used in removal of lignocellulose-containing dung cladding from the animal hides (Auer et al. 1999) and, more importantly, in replacing environmentally precarious chrome-based tanning agents in the process of leather making (Thanikavvelan et al. 2004, 2005) and in turning natural polyphenols in the animal skins or added phenolic compounds into lightfast pigments (Covington et al. 2005, Surpano et al. 2005). Of pharmaceutical potential as antioxidants are polymers produced from plant flavonoids by laccase (Uyama 2007). Whilst the so far mentioned applications are all possible, they essentially play no or only a minor role in current industrial processes. This is different from the situation in the pulp and paper and the textile industry (Riva 2006, Schäfer et al. 2007).

In the pulp and paper industry, enzymes are introduced to substitute environmentally harmful chemicals or high energy consuming processes. Laccases were shown to delignify kraft pulp in the presence of a mediator, thus displacing the conventional chlorine bleaching (Archibald et al. 1997, Bajpaj 2004). Mediator compounds oxidised by the enzyme may act directly on lignin and other structures that are not laccase substrates (Guiterrez et al. 2006). Efficiency of bleaching by the laccase-mediator system was demonstrated in a pilot scale which allowed to save about 24% of chlorine dioxide during the milling process (Grönqvist et al. 2003). Most of the effective mediators are synthetic chemicals containing a N-OH functional group but also potent natural compounds, e.g. 3-hydroxyanthranilate and 4-hydroxybenzoic acid, have been described (Eggert et al. 1996a, Johannes & Majcherczyk 2000, Annunziatini et al. 2005). The use of the plant phenols acetosyringone and syringaldehyde in combination was reported to enable an over 15% increase in final brightness of paper pulp together with a similar decrease in the

kappa number, a value correlated to the residual lignin content (Camarero et al. 2007). Similarly good results were obtained with 1-hydroxybenzotriazole (Ibarra et al. 2006). Beside this application in bleaching, laccases can also be used to decolourise chromophores from kraft mill bleachery effluents (Archibald et al. 1990, Munari et al. 2007, Pedroza et al. 2007), in bleaching as well as in colouration of textile fibres (Basto et al. 2007, Kim et al. 2007, Ren & Buschle-Dillier 2007), and in decolourisation of recalcitrant textile dyes in waste waters (Svobodová et al. 2003, 2008, Sharma 2005, Šušla & Svobodová 2006). Noteworthy is the application of laccases in refining jeans: surplus of the blue indigo dye is degraded by the enzymes. As result of such treatment, the fabric has a stone-washed appearance without the negative stressing on the fibre structures occurring in conventional stone washing (Campos et al. 2001, Pazarlioglu et al. 2005). Regarding textiles, laccases in combination with cellulases are applied in detergents for efficient cleaning of stains on textiles and other fabric materials, particularly in industrial laundries (Galante & Formantici 2003).

Production of wood composites from fibres and particles by means of environment-friendly bio-adhesives is another compelling area for applications of laccases. In order to replace the commonly used binders on petrochemical basis (urea-formaldehyde, phenol-formaldehyde, phenyldiisocyanate; see Chapter 15 of this book), lignin on fibre surfaces (lignin of the middle lamellae) is activated by laccases through oxidative enzymatic reactions yielding lignin radicals for subsequent polymerisation reactions (Kharazipour et al. 1997, 1998, Hüttermann et al. 2001, Felby et al. 1997, 2002, 2004; for an extensive presentation see Chapter 18 of this book).

Due to their broad oxidative capabilities, laccases have been proposed for applications in bioremediation. Diverse xenobiotic compounds like chlorophenols (Leontievsky et al. 2001), synthetic dyes (Rodriguez et al. 1999), herbicides (Mouglin et al. 2000), non-phenolic aromatic hydrocarbons (Johannes et al. 1996) and many other toxic organic pollutants (Couto & Herrera 2006, Kilaru 2006, Nyanhongo et al. 2007) were shown to be oxidised by laccases. Furthermore, the use of mediators greatly extends the range of degradable substances (Majcherczyk et al. 1999). Besides, laccases can also catalyse oxidative coupling reactions leading to polymeric products (Uyama & Kobayashi 2002, 2006; see also Chapter 18 of this book). In soils, laccases can copolymerise xenobiotic substances and bind them to humic material making them unavailable to interact with the biota and also preventing their movement via leaching (Bollag 1992). Generally for soil bioremediation, application of fungal cultures producing laccases appears to be most practicable (Rama et al. 2001, Hestbjerg et al. 2003, Novotný et al. 2004, McErlean et al. 2006, Steffen et al. 2007). Contaminations in solid materials can be addressed by solid state fermentation (SSF) with white-rot fungi (see Chapter 19 of this book). Living fungi producing laccases might also be of use for bioremediation of contaminated liquids in suitable bioreactors (Kasinath et al. 2003, Ryan et al. 2005,

Diano et al. 2007; see also Chapter 19 of this book). Furthermore, experiments are performed to immobilise the enzyme onto suitable matrices for clearance of contaminated waste waters (Hu et al. 2007, Lu et al. 2007).

Enzyme immobilisation is an integral part in the production of laccase-based biosensors constructed to measure concentrations of phenolic compounds in waste waters and other samples taken from the environment, to determine the phenol contents in wines, teas, and other beverages, to define concentrations of compounds in pharmaceutical formulations and medical samples, to screen plant extracts for phenolic compounds, and to detect toxic phenolic chemicals including xenobiotic compounds in various other materials (Ghindilis et al. 1992, Yaropolov et al. 1994, 2005, Minussi et al. 2002, Leite et al. 2003, Gomes et al. 2004, Jarosz-Wilkolazka et al. 2004, Gamella et al. 2006, Vianello et al. 2006; see also Chapter 12 of this book). In addition, the involvement of molecular oxygen in laccase catalysis allows to measure oxygen with such biosensors, even at rather low levels (Gardiol et al. 1996). Biosensors may be self-powered (Katz et al. 2001). Co-immobilisation of laccases with redox polymers in cathodes allows to construct bio-fuel cells that generate bioelectric power from enzymatic oxidations (Tayhas & Palmore 2004, Davis 2007).

Peroxidases

In contrast to laccases, the application of peroxidases for degradation/modification of lignocellulose in biotechnological processes is limited (Piontek 2002) by the demand on controlled amounts of H_2O_2 (compare enzyme reaction schemes in Fig. 2).

Application of peroxidases and hydrogen peroxide was suggested in the seventies for binding of panel boards (Nimz et al. 1972, 1976). Treatment of wood fibres by peroxidases and hydrogen peroxide allowed producing medium density fibreboards (MDF) in comparable quality to boards obtained with laccase (Kharazipour et al. 1998). Unlike laccases, peroxidases, however, were never applied on a technical scale. Requirement of controlled amounts of H_2O_2 during the reactions and optional addition of veratryl alcohol remained to be serious barriers in large scale industrial usage of peroxidase (Hüttermann et al. 2001; further reading in Chapter 18 of this book).

Similarly to laccases, also peroxidases have the potential to act in pulp bleaching (de Carvalho et al. 1998, Moreira et al. 2003, Sigoillot et al. 2005). Laboratory scale experiments showed for example that bleaching of kraft pulp by MnP reduced the kappa number by 50%, thereby reducing the extent of the final chlorine treatment (e.g. Kondo et al. 1994, Moreira et al. 1997, Sigoillot et al. 1997) and resulting in demethylation of lignin (Paice et al. 1993). Nevertheless, the requirement for H_2O_2 again limits the application of peroxidases in bleaching (Paice et al. 1995).

Furthermore, LiP, MnP, and versatile peroxidase as effective lignin degrading enzymes are, as laccases, able to oxidise a number of organic compounds such as polycyclic aromatic hydrocarbons and various recalcitrant textile dyes that can be harmful to the environment (e.g. see Young & Yu 1997, Gunther et al. 1998, Kim et al. 1998, Ferreira et al. 2000, Couto et al. 2002, Verma & Madamwar 2002, Mielgo et al. 2003, Mohorcic et al. 2006, Ferreira-Leitoa et al. 2007, Tinoco et al. 2007). Whilst application of enzyme solutions to such compounds have shown their effectiveness, living fungal cultures are better applied for bioremediation of contaminated soils and other materials and for purification of aqueous solutions since they produce the necessary enzymes together with H_2O_2 due to the action of the auxiliary enzyme machinery of lignocellulose degradation (Pointing 2001, Gianfreda & Rao 2004, Tortella et al. 2005, Šušla & Svobodová 2006). Concerted actions with other lignolytic enzymes generated by the fungi can further improve handling of substances that otherwise would be difficult to degrade (Nyanhongo et al. 2007).

Screening for new enzymes

New versions of well known enzymes

The above addressed example of cellulase required at tenfold more activities for the same price to make biofuel production from lignocellulosic material economical (Sheehan & Himmel 2001) provoked large screening programmes for new and better enzymes (Zhang et al. 2006). By reasons i. that by industrial interests high producing strains are not freely available to everybody, ii. that enzymes are not produced in such high amounts as required for industrial application, or iii. that available enzymes are not as ideal for specific applications, screenings are also ongoing for the other main types of fungal enzymes acting in lignocellulose degradation [e.g., see recent publications of Kuiskinen et al. (2004), Krogh et al. (2004), Dhoubi et al. (2005), Kabe et al. (2005), Chairattananakorn et al. (2006), Guimaraes et al. (2006), Songulashvili et al. (2007)].

Interesting enzymes that come up from such screens might be produced in suitable fermentation processes from their natural hosts. Yields may however not always be satisfying. If the proteins are from a new species, fermentation conditions might have to be established from scratch. As alternative strategies, recombinant enzyme production from heterologous hosts with running fermentation processes might be approached, once the genes for enzymes of interest have been identified and cloned (further reading in Chapter 19 of this book).

Many biotechnological applications call for relatively pure and/or large quantities of enzymes. In order to meet these demands, the genes of the enzymes in question are overexpressed in heterologous hosts like ascomycetous yeasts (*Saccharomyces cerevisiae*, *Pichia pastoris*) or filamentous ascomycetes (*Aspergillus* spp., *Trichoderma* sp.). This approach is successfully applied with hydrolytic enzymes from

ascomycetous species (Kulkarni et al. 1999), but was repeatedly shown to be problematic for enzymes from wood-decaying basidiomycetes due to differences in protein glycosylation (e.g. see Schneider et al. 1999, Otterbein et al. 2000, Larrondo et al. 2003a, Liu et al. 2003, Sigoillot et al. 2004). Since the latter group of organisms has several enzymes with unique capabilities, alternative expression systems had to be established. One strategy is to overexpress the basidiomycetous genes in basidiomycetous species. This approach was shown to be successful for the expression of basidiomycetous laccase genes in the *P. cinnabarinus* (Alves et al. 2004), *T. versicolor* (Kajita et al. 2004), and *C. cinerea* (Kilaru 2006, Kilaru et al. 2006b; further reading in Chapter 19 of this book). Furthermore, peroxidases from *P. ostreatus* and *T. versicolor* have been (over-)produced upon gene transformation in *P. ostreatus* (Irie et al. 2001, Tsukihara et al. 2006a,b), *T. versicolor* (Yeo et al. 2007), and *C. cinerea* (Ogawa et al. 1998).

Next to screening for enzyme activities in fungal isolates, it is also possible to identify gene sequences for potential enzymes by molecular DNA screening techniques (Pointing et al. 2005). With the number of identified and sequenced genes of enzymes involved in the degradation of lignocellulose growing steadily, recombinant approaches for the production of biotechnologically interesting enzymes become practice. The progress achieved in gene identification is exemplified by the number of fungal multi-copper oxidase genes known a few years ago (about 15; Leonowicz et al. 1999a) and now (258 genes for multi-copper oxidases including 165 genes for laccases *sensu stricto*; Hoegger et al. 2006).

Unique resources for fast gene identifications are presented by genome sequencing projects. Recent sequencing of fungal genomes (for the continuously growing lists of sequenced fungal genomes see <http://www.fgsc.net/outlink.html>, <http://www.broad.mit.edu/annotation/fungi/fgi/>, and <http://genome.jgi-psf.org/index.html>) uncovered that the basidiomycetes possess much larger reservoirs of genes for enzymes with possible functions in lignocellulose degradation than one might have thought off. Previously, experimental evidence in *C. cinerea* argued for the occurrence of at least one functional laccase protein and three different genes with cDNAs (Yaver et al. 1999). For first three and then five other genes, genomic DNA sequence was available (Bottoli et al. 1999, Hoegger et al. 2004). Still, it was a surprise to finally find a total of 17 different laccase genes in the genome of this fungus (Kilaru et al. 2006a). Recombinant DNA technology revealed that at least 13 of these genes can give a functional protein and first analysis of protein properties including substrate preferences suggests that these are not merely redundant in function. Different enzymatic characteristics - particularly different substrate preferences - may make them suitable for different types of applications (Kilaru 2006). It is interesting to note that, quite contrary to *C. cinerea*, no conventional laccase gene was found in the genome of the white-rot fungus *P. chrysosporium* (Larrondo et al. 2004). Of its five multi-copper oxidase genes, one is homologous to genes for Fet3-like ferroxidases and one of four closely related

genes has been shown to exhibit Fe^{2+} -oxidising activity combined with a low lac-case activity which however is expected to be not relevant (Hoegger et al. 2006, Larrondo et al. 2003b, 2007).

From the insight of the few basidiomycete genomes we have obtained so far, it appears that also the furnishing of genes for other proteins with functions in wood degradation is very variable from fungus to fungus (see also below). Ten known LiP genes in *P. chrysosporium* (Gaskell et al. 1994) have for example been defined in the genome in addition to five MnP genes (amongst the three formerly known genes *mnp1*, *mnp2*, *mnp3*), a partial *mnp*-like sequence and a gene *nop* for a novel peroxidase (Martínez et al. 2004, Larrondo et al. 2005). In contrast, *C. cinerea* has only one gene for a classical peroxidase (U. Kűes, unpublished observation) which is the well characterized enzyme CIP (see above). The novel peroxidase encoded by gene *nop* in *P. chrysosporium* is closest related to *C. cinerea* Cip (Larrondo et al. 2005). Substrate ranges and catalytic properties of this *P. chrysosporium* enzyme have still to be established.

Novel types of enzymes with conventional biochemical functions

With genome sequencing programmes, research strikes a new path to identify novel types of enzymes. Specific domains from described proteins can help with suitable computer programmes to predict protein functions from so far unknown genes (Tatusov et al. 2003, Bateman et al. 2004), even if they are only from small, rare protein families. In the following, some interesting novel types and groups of enzymes from white-rot and litter-degrading basidiomycetes are discussed that may play roles in degradation of lignocellulosic materials and/or accompanying detoxification processes and that in the future could become versatile biotechnological tools.

Heme-thiolate haloperoxidases are poorly studied enzymes that share catalytic properties with classic fungal peroxidases, cytochrome P450 monooxygenases, and catalases. Next to classic peroxidase reactions, these enzymes perform halogenations and oxygenation of certain substrates (Hofrichter & Ullrich 2006). Two such unusual peroxidases have recently been described from *Agrocybe aegerita* and *Coprinellus radians*, respectively, basidiomycetes that occur on hardwoods. The enzymes oxidise aryl alcohols at a pH around 7.0 using H_2O_2 as electron donor, halogenate phenols, and selectively hydroxylate naphthalene – whether these enzymes participate somehow in lignin or humus degradation or whether they are rather detoxifying enzymes is not known so far (Ullrich et al. 2004, Ullrich & Hofrichter 2005, Ahn et al. 2007). Complete gene sequences are missing but sequences of a few peptides of the two enzymes are available including one with a cysteine residue supposed to act in heme-binding (Ahn et al. 2007). The peptides allowed searching for potential haloperoxidase genes in the available basidiomycetes genomes and the peptide with the cysteine gave positive hits. Accordingly, in

both, *C. cinerea* and *P. chrysosporium*, there are a few candidate genes for proteins for a new fungal peroxidase family 2 with currently only two recognised members that do not have similarity to classical peroxidases (U. K  es, unpublished observation). Classical peroxidases react at low pHs in the range of 2.5 to 5.5 (e.g. see Kirk & Farrell 1987, Bekker et al. 1992, Bonnen et al. 1994, Koroleva et al. 2002, Wang et al. 2002, Baborova et al. 2006) and the family 2 peroxidase might have evolved for functioning under more alkaline environments (Hofrichter & Ullrich 2006). With the unusual pH range and, moreover, the additional aromatic peroxygenation activity, family 2 peroxidases offer unique possibilities for biotechnological applications, e.g. in hydroxylation of herbicides (Ahn et al. 2007, Ullrich & Hofrichter 2007).

Homologues to a gene from *Termitomyces albuminosus* of another unusual manganese-independent peroxidase with a pH activity in the extreme acidic range (pH 2.3) and a selective substrate range excluding veratryl alcohol (Johjima et al. 2003) are present in the genome of *C. cinerea* but not in the genome of *P. chrysosporium* (U. K  es, unpublished observation). Related novel peroxidases have been described in *Thanatephorus cucumeris* (Sato et al. 2004), in *P. ostreatus* (Faraco et al. 2007), and possibly in *Trametes hirsutus* (Shin & Lee 2000). These enzymes belong to the DyP family of atypical peroxidases named after the dye-decolourising activities of a first described peroxidase from the ascomycete *Galactomyces geotrichum*. DyP family peroxidase bind heme but lack the heme-binding region conserved among the plant peroxidase superfamily including the fungal class II family (Sato et al. 2004, Faraco et al. 2007). At least the *T. cucumeris* enzyme has also dye-decolourising activities (Sugano et al. 2006).

As a challenge to synthetic chemistry, actively ongoing genomic research however focus currently on genes for intracellular heme-containing cytochrome P450 monooxygenases able to perform selective hydroxylation of aromatic compounds (Ullrich & Hofrichter 2007). Most striking in the genome of the white-rot *P. chrysosporium* is the number of more than 150 genes for cytochrome P450 monooxygenases classifying in 11 different families and 23 subfamilies (Mart  nez et al. 2004, Doddapaneni et al. 2005, Yadav et al. 2006) which presents an immense potential for biotransformations of organic compounds including xenobiotic chemicals (Matsuzaki & Watanishi 2004, Teramoto et al. 2004). Estimates for the family of P450 cytochromes in *C. cinerea* have not yet been published but over 50 different entries for potential enzymes are easily found in the NCBI (National Center of Biotechnology Information) GenBank database (<http://www.ncbi.nlm.nih.gov/sutils/blink.cgi?pid=3721844>; U. K  es, unpublished observation).

The addressed examples of gene families for enzymes with potential lignocellulose degradation and xenobiotic compound detoxification functions document how modern genomics can help to expand the pool of enzymes available for biotechnological approaches. In order to define conditions of high level protein production, identified genes might be studied in expression individually or, more efficiently, by fixing batches of gene probes in an assorted manner on micro-ar-

rays for hybridisation with DNA probes of transcripts from expressed genes (Doddapaneni & Yadav 2005). Alternatively, individual genes might be subcloned and expressed under control of different promoters in the original or a different host in order to produce, biochemically characterise, and eventually utilise the enzymes (Matsuzaki & Wariishi 2005, Kilaru 2006, Kilaru et al. 2006b; see Chapter 19 of this book).

Novel types of enzymes of unknown functions in the free fungal secretome

From an estimated total of 10,048 genes in the *P. chrysosporium* genome (prediction v2.1 of the Department of Energy's Joint Genome Institute's Genome Portal; www.jgi.doe.gov/whiterot), the products of 769 genes - amongst peroxidases and other oxidoreductases, peptidases, glycoside hydrolases and esterases-lipases - are predicted to be secreted. This number might however be underestimated by the tendency of computer programmes to wrongly predict the N-termini of proteins where in secreted proteins usually the secretion signals reside. About half of the proteins predicted to be secreted have yet no assigned function. Expressed under ligninolytic and cellulolytic conditions, it is assumed that at least some of these may also be involved in lignin degradation (Vanden Wymelenberg et al. 2006a, Kersten & Cullen 2007). Their industrial potential will probably be realised when the genes are cloned and expressed in large quantities. With establishing their functions, yet not foreseeable applications might arise.

From the saprophytic dung fungus *C. cinerea*, estimates for secreted enzymes have not yet been published. Our own analysis of the computer-annotated genome (Broad release of gene set version 1.0; NCBI Genbank accession number AAC01000000) identified 1,769 potential secreted proteins in a total of 13,562 proteins (A. Majcherczyk, unpublished). Once a sequence of a fungal genome is established and reasonably well annotated, it becomes possible to identify individual proteins secreted into the environment (Fig. 7). Ongoing studies on protein identification by mass-spectroscopy confirmed so far genes for 76 proteins secreted into liquid culture medium of *C. cinerea*. Amongst these proteins are β -glucosidases, β -1,3-glucanases, glucoamylases, metalloproteases, serine proteases, and endopeptidases required for substrate degradation (see Fig. 4). Some other proteins are totally new and have not yet been described in function (A. Majcherczyk et al. unpublished).

Similar proteomics approaches revealed also in the white-rot *P. chrysosporium* a rich diversity of secreted enzymes for substrate degradation including various LiPs, peptidases, lipases, glycosyl hydrolases, carboxylesterases, and a mannose-6-phosphatase when grown under C- and N-limitation (Vanden Wymelenberg et al. 2006a), glycosyl hydrolases, endoglucanases, exocellobiohydrolases, endoxylanases, an α -galactosidase, an acetyl xylan esterase, and an α -L-arabinofuranosidase

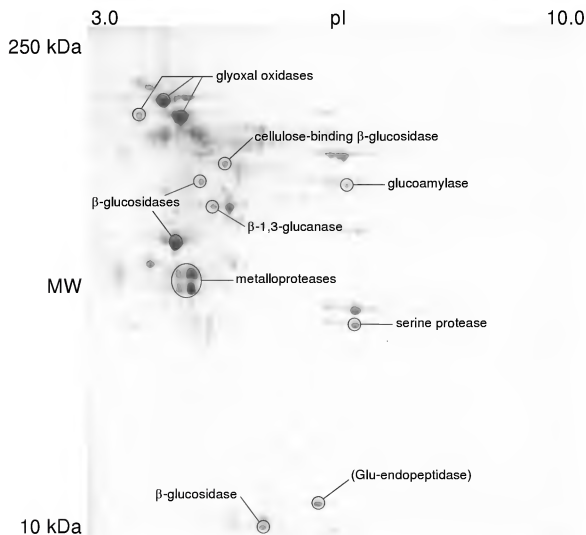


Fig. 7 2D-gel of proteins from a culture supernatant of *Coprinopsis cinerea*. The fungus was grown at 37°C for 9 days in liquid medium (Kjalke et al. 1992), the culture liquid separated from the mycelium, and proteins concentrated for gel-electrophoresis according to standard protocols. Tryptic digests of individual proteins were analysed by mass-spectroscopy and results compared with a sequence database containing *C. cinerea* proteins as predicted from genome annotation (Dwivedi 2006). Note that predicted functions of positive hits in all cases were enzymes with functions in degradation of organic polymeric substrates

when grown on cellulose (Vanden Wymelenberg et al. 2005), and a cellobiohydrolase, a cellobiose dehydrogenase, a β -glucosidase, an α -galactosidase, an endopolygalacturonase, an exocellobiohydrolase, a β -endoglucanase, a laminarinase (endo-1,3(4)- β -glucanase), a xylanase, a glucan 1,3- β -glucosidase, and a LiP when grown on oak wood chips (Abbas et al. 2005).

Doing the same type of identification with proteins from culture medium of other white-rot species is currently less efficient since restricted to proteins or genes known by experimental work such as the different types of phenol oxidases encoded by genes of *T. versicolor* and *P. ostreatus*. Often in these fungi, isoenzymes are secreted (Homolka et al. 1997, Giardina et al. 2000, Palmieri et al. 2000, Cohen et al. 2001, Dwivedi 2006). Whilst activity staining of enzymes in 1D and 2D gels can identify different iso-enzymes e.g. of laccases (Fig. 8), proteomic analysis can assign them to specific genes or define them as isoforms coming from the same gene (Dwivedi 2006). Using different types of laccase substrates, in-gel staining can differentiate between isoenzymes in their substrate spectra and overall quality of activities (Lang 2004, Dwivedi 2006). Such difference in activities as observed in the cited studies for laccases in different white-rot fungi demonstrate why typically more than one protein is produced – with variations in substrate specificities, an organism is much more versatile to react on the various substrates and compounds possibly offered by the environment. The results from the proteomic approaches on freely secreted proteins in *C. cinerea* and *P. chrysosporium* show that expression of multiple isoenzymes is also verified for other types of enzymes acting in substrate utilisation (see above and Fig. 7). Overall, new enzymes are discovered in the group of proteins freely secreted into the medium but few with entirely new functions (see above).

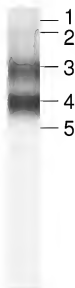


Fig. 8 Native 1D-gel of a culture supernatant of *P. ostreatus* var. *florida* strain F6 (Homolka et al. 1997) stained according to Dicko et al. (2002) to detect laccase activity. The various bands (1 to 5) present different enzymes or enzyme isoforms (note the different strength of the bands indicating different levels of enzyme activities that might be caused by different amounts of protein and/or different efficiencies in interactions with the substrate)

Novel types of secreted enzymes associated with the fungal cell walls

The **secretome** (= the total of all secreted proteins of an organism) of fungi subdivides into proteins that are freely released into the environment, proteins that associate with the cell walls and proteins that remain in the periplasmic space between outer cell membrane and cell wall. So far, little, respectively nothing at all is known for most basidiomycetes on proteins associated to the cell wall and present in the periplasmic space.

Compared to the freely secreted and thus movable proteins, identification of proteins from the cell wall and the periplasmic space is more challenging since the enzymes have to be freed from their respective compartments in sufficient amounts before such analysis can be performed (Rast et al. 2003, Dwivedi 2006). Likely also because of the difficulties to obtain suitable protein fractions, enzymes localised in the hyphal sheaths and cell walls have until recently mostly been neglected (Rast et al. 2003, Valášková & Baldrian 2006a). Using immunological methods with antibodies raised against known enzymes, presence of enzymes has been demonstrated within the periplasmic space of hyphae, in association with the cell membrane, with periplasmic vesicles, the fungal cell walls, and/or extracellular sheaths. Examples are laccase and peroxidases in *P. radiata* (Daniel et al. 2004), laccase in *Rigidoporus lignosus* and other white-rot fungi (Nicole et al. 1992, 1993, Daniel 1994), peroxidases in *P. chrysosporium* (Daniel et al. 1989, Ruel & Joseleau 1991), aryl alcohol oxidases in *Pleurotus eryngii* (Barrasa et al. 1998), pyranose oxidase in *P. chrysosporium* and *Oudemansiella mucida* (Daniel et al. 1994), and cellulolytic enzymes in *Volvariella volvacea* (Cai et al. 1999). Furthermore, enzymatic tests revealed laccase-like activities in cell walls of *T. versicolor*, *I. lacteus*, and *C. cinerea* (Svobodová et al. 2003, 2008, Dwivedi 2006, M. Navarro-González, personal communication; Fig. 9). Significant amounts of 1,4- β -glucosidase, cellobiohydrolase,

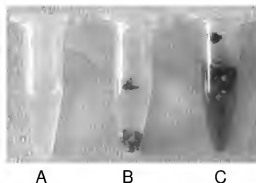


Fig. 9 Mycelium of *Ipex lacteus* is stained greenish-blue by a laccase-like activity oxidising the colourless substrate ABTS [2,2'-azino-di-(3-ethylbenzothiazolin-6-sulphonic acid)]. A. 100 mM Na-acetate buffer, pH 5.0 + 5 mM ABTS. B. Lyophilised mycelium in 100 mM Na-acetate buffer, pH 5.0. C. Lyophilised mycelium in 100 mM Na-acetate buffer, pH 5.0 + 5 mM ABTS. All tubes were incubated for 1 min at room temperature

1,4- β -xylosidase, and 1,4- β -mannosidase activities in straw cultures of *P. ostreatus* (66% of the total activity), *T. versicolor* (35%), and *Piptoporus betulinus* (8%) were found cell-wall bound, much more than in mycelium grown in liquid culture in no direct contact of lignocellulosic plant material (Valášková & Baldrian 2006a). Within straw and wood, the fungal cell walls and the surrounding hyphal sheaths mediate a direct contact to the plant cell wall (Nicole et al. 1993, 1995, Barrasa et al. 1998, Abu Ali et al. 1999, Daniel et al. 2004). In such case, there is no need for free secretion of fungal enzymes. Cell wall-associated enzymes are directly at the place where they are needed for action. It is of further advantage that enzymes are stabilised in the immobilised, i.e. cell wall-associated state (Svobodová et al. 2003, 2008, Dwivedi 2006). Research has shown that cell-wall bound enzymes could serve in waste water treatment for degradation of recalcitrant textile dyes, polycyclic aromatic compounds, and other recalcitrant compounds (Swamy & Ramsey 1999, Svobodová et al. 2003, 2008, Raghukumar et al. 2006). Currently, it is not known whether these active cell wall-associated enzymes are identical to the proteins found in liquid culture media or whether they are new enzyme species. Identification of the individual cell wall-associated enzymes will allow to clone the corresponding genes and to overexpress them for better characterization.

Techniques known from analysis of cell wall-associated proteins from ascomycetes could not simply be transferred to release proteins from the hyphal sheaths and cell walls of filamentous basidiomycetes. Much effort was required to develop suitable methods to obtain protein fractions from the extracellular hyphal sheath, SDS (sodium dodecylsulphate)-extractable non-covalently bound cell wall proteins, and the covalently bound cell wall proteins that are free of contamination of intracellular proteins (Dwivedi 2006). Ongoing protein identification assessment promises to detect new proteins amongst already known enzymes (Dwivedi 2006, A. Majcherczyk, unpublished).

Conclusions

The process of wood degradation by basidiomycetes has been intensively studied in the last 30 years and led to significant advances in understanding of wood structures and enzymes involved in their degradation. Promising technological applications of enzymatic lignin degradation performed by white-rots strongly pushed the research focus towards this group of fungi. Although numerous enzymes implicated in the wood biodegradation have been identified and studied, the exact mechanisms of fungal wood degradation and the variability of these mechanisms are still not fully understood. Interactions of known and new enzymes as well as low molecular weight peptides with non-enzymatic oxidative systems are at present a subject in wood degradation research. A better understanding of these interactions and mechanisms will help in the search of enzymes optimised (natural or recombinant) for specific biotechnological applications. New methodical approaches of

ferred by genomics and proteomics will lead this research into new directions and, very likely, new versions of known enzymes as well as novel types of enzymes will both be discovered.

The non-enzymatic lignin-degrading system of brown-rots able to generate oxygen radicals was also recognised as an important wood-degrading pathway in the last decade (Martínez al. 2005). Such fungi have their value in bioremediation of CCA (copper, chromium, arsenic) or CCB (copper, chromium, boron) treated wood waste by complexing the metals through oxalic acids (Samuel et al. 2003). Oxalate decarboxylases of these fungi converting oxalic acid to formic acid and carbon dioxide and providing a buffered environment facilitating the wood decay process have found some interest in the past (Micales 1997) but otherwise enzymes in this group of fungi found so far little interest. A first genome of a brown-rot has recently been released to the public (*Postia placenta*, <http://genome.jgi-psf.org/Pospl1/Pospl1.home.html>) and with *Serpula lacrymans* a second brown-rot genome is pending for sequencing (<http://www.jgi.doe.gov/sequencing/allinoneseqplans.php>). Comparing these genomes with the available and pending genomes from white-rots (*P. chrysosporium*, pending; *Heterobasidion annosum*, *Phanerochaete carnosa*, *P. ostreatus*, *Schizophyllum commune*), dung fungi (*C. cinerea*, pending; *A. bisporus*) and ectomycorrhizal basidiomycetes (*Laccaria bicolor*, pending; *Paxillus involutus*) (<http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html>; <http://www.jgi.doe.gov/sequencing/allinoneseqplans.php>) will be another interesting task for the future and should help to come to a better understanding of the different strategies of wood degradation by fungi.

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18. Enzymatically Modified Wood in Panel Board Production

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Introduction

The production of wood composites such as fibre- or particle boards or OSB (oriented strand boards) follows always the same basic process. First, solid wood is fragmented into smaller pieces, i.e. fibres, chips or strands. These are supplemented with a binder and pressed under heat to form a wood-like material (wood composites) (Malony 1993). Currently in industrial production, the binders added to the wood are of synthetic nature and most likely either urea-formaldehyde, phenol-formaldehyde, or organic isocyanates (see Chapter 15 of the book). However, there are plenty attempts to replace these synthetic glues by bio-based

adhesives (Kharazipour & Hüttermann 1992, 1998, Pizzi 2000, 2006; see Chapter 16 of this book). Another environmental friendly strategy is to enzymatically activate the natural bounding forces of wood fibres. This is particularly interesting for fibreboard production (Hüttermann et al. 2001, Mai et al. 2004). There are two principle ways of obtaining enzymatically activated fibres and wood particles. The wooden material might be incubated with isolated enzymes or it might be incubated with living white-rot fungi that produce suitable enzymes during cultivation (see Chapters 17 and 19 of this book for further information on white-rot fungi).

Enzymes in lignin activation

Wood is mainly composed of lignin, hemicelluloses, cellulose, and pectin. These polymers are not evenly distributed in the cell walls of the fibres but in variable amounts and combinations assigned to different cell wall layers (see Fig. 1). The outer layer, the middle lamella (ML) connecting the neighbouring cells, consists largely of pectin as cementing substance and of lignin (up to 80% and more) mediating stiffness. The primary cell wall (PW) is made up by a matrix of pectin and primarily hemicellulose in which cellulose fibres are incorporated in almost random manner (dispersed texture). The secondary cell wall (SW) is composed up to 94% of cellulose. Forming the largest part of the fibre cell wall, it is divided into three different layers. The S1 layer lies next to PW and consists of cellulose fibrils that are arranged in parallel roughly transversely to the longitudinal axis of the cell

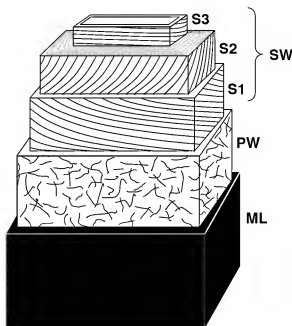


Fig. 1 An idealised model of the cell walls of wood fibres modified from Schwarze et al. (2000) after Kerr & Bailey (1934)

(helical texture). The S2 layer, being several micrometre thick, forms 74-85% of the whole cell wall and is composed of several concentrically arranged lamellae of parallel, helical-oriented, and mechanically strong cellulose fibrils (helical texture) that run more or less longitudinal to the cell axis and are embedded in a film of hemicellulose providing structural flexibility. The S2 layers mediate tensile strength to the fibres through incorporation of lignin in between cellulose and hemicellulose. The thin inner layer of the secondary cell wall (S3 - also referred to as tertiary cell wall, TW) is formed by parallel-oriented or slightly scattered cellulose fibrils in a texture resembling somewhat that of the primary cell wall. Also this layer exhibits lignification - in conifers even more than what occurs in the S2 layer - thereby conferring a higher resistance against fungal decay to the S3 layer (Schwarze et al. 2000, Sitte et al. 2002, Fengel & Wegener 2003). The different types of decay fungi (white-rot, brown-rot, soft-rot) attack primarily the middle lamellae and the carbohydrate-rich S2 layers of the fibres. White-rot fungi specifically attack the lignin in these layers, either selectively or simultaneously with the cellulose, by producing enzymes such as laccases and different types of peroxidases which react with lignin (Leonowicz et al. 1999, Schwarze et al. 2000; Chapter 17 of this book).

Wood can efficiently be defibrated by steam explosion through application of pressured steam at high temperature such as in the Masonite-process or in the Asplund-process which includes an additional mechanical milling step (thermo-mechanical pulping, TMP) (Lampert 1966, Kotka & Ahmed 1997). Both techniques are applied in producing the raw material for fibreboard manufacture (see Chapter 15 of this book for further details). In these processes, the fracture zones of fibres are the lignin-rich middle lamellae that connect the fibres in assembled wood (Johannson et al. 1999; Fig. 2). Temperature determines this fracture zone (Koran 1968, Dorris & Gray 1978, Mjöberg 1981). Dry lignin has a glass-transition point

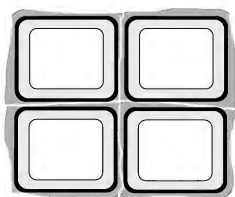


Fig. 2 In high-temperature and high-pressure defibration of wood chips, the fracture zone between wood fibres is usually within the lignin-rich middle lamellae (dark grey) whilst primary (black) and secondary cell walls (light grey) remain intact (Dorris & Gray 1978, Mjöberg 1981)

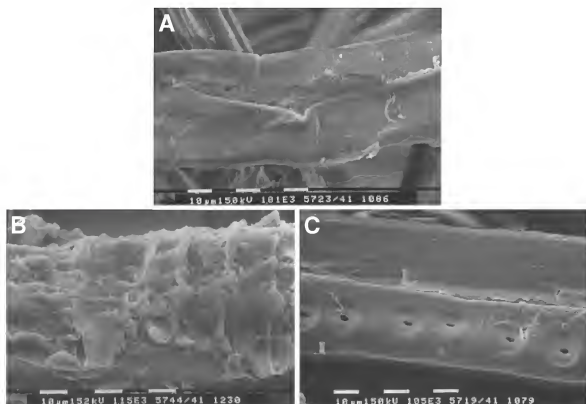


Fig. 3 Surface of wood fibres before and after laccase treatment (from Kharazipour & Hüttermann 1993 and Kharazipour 1996). A. A crust of lignin is formed on the fibre surface through plastifying of the middle lamella lignin during high-temperature and high-pressure defibration of wood chips and subsequently cooling down to room temperature. B. The plastified crust is loosened by short-term enzymatic treatment. C. The lignin crust is fully removed after long-term incubation (bleached fibre)

(T_g) between 130 and 190°C where it begins to melt. Moisture lowers the T_g . In the wet condition (moisture > fibre saturation point), the T_g is in the range of 60 and 100°C (Goring 1963). In order to obtain fibres that are most suitable for board production, defibration is performed at high refining temperatures, typically above T_g at 170°C to 190°C (Widsten et al. 2003, 2004). Fibres obtained from defibration processes at such high refining temperatures have an altered surface. The heat causes plastification of the lignin in the middle lamellae and, in consequence, separation of the fibres during the defibration process. Upon cooling down, the lignin hardens again and forms a glassy crust on the surface of the fibres (Mjöberg 1981, Zavarin 1984, Wagenführ 1988, Kharazipour & Hüttermann 1993; Fig. 3A). Furthermore, heat together with pressure triggers chemical alterations to lignin as well as to hemicellulose (Sun et al. 1999). Lignin is modified e.g. by hydrolytic cleavage of ether bonds and by homolytic cleavage of covalent bonds (Hon 1983, Lee & Sumimoto 1990, Widsten et al. 2002a), resulting in the generation of low molecular weight lignin fragments and relocation of lignin from

inner cell wall layers to the fibre surface (Zavarin 1984, Widsten et al. 2001). Holmolytic cleavage of β -O-4 ether bonds in fibre lignin generates phenoxy radicals that may undergo various further chemical reactions affecting i. the molecular size distribution and content of functional groups of the lignin polymers and ii. the generation of water extractable material enriched in hemicellulose and aromatic substances of lower degree of etherification and richer in phenolic hydroxyl groups (Lee & Sumimoto 1990, Widsten et al. 2001, 2002a). Hardwood lignins with high syringyl content (see Chapter 7 of this book for discussion of chemical compositions of lignins) have a lower Tg as compared to other lignins (Olsson & Salmen 1992). Accordingly under comparable conditions, hardwood syringyl-type lignin is more extensively depolymerised than the guaiacyl-type lignin (Widsten et al. 2002a). A rise in temperature during defibration increases lignin depolymerisation both in hardwood and softwood and it increases the formation of reactive water-extractable material (Widsten et al. 2001, 2002a).

The lignin on the fibre surfaces and the released reactive water-extractable material mediates some degree of self-adhesion to the fibres. Pressing at high forces and temperatures for long periods can create water-resistant bonds (Klauditz & Stegmann 1955, Back 1987, Unbehaun et al. 2000). Pyrolytic degradation of hemicellulose sugars and thermal conversion to furan products have been discussed to contribute to the bonding and increase of free reactive sites on the aromatic ring of lignin units and to auto-condensation of lignin (Zavarin 1984, Ellis & Paszner 1994, Tjeerdsma et al. 1998, Rowell et al. 2002). Boards formed by just pressing fibres have however unfavourable properties: they show high thickness swelling in presence of water and have poor mechanical properties (Anglès et al. 1999, Unbehaun et al. 2000). The glassy lignin crust on the fibres as such is little reactive and forms a barrier to strong fibre-fibre bonding (Wagenführ 1988, Unbehaun et al. 2000). Optimising the conditions of physical and chemical fibre pretreatment and changing board production conditions for thermal reactivation of the fibre surface lignin can lead to better bonding and, in turn, to improved board properties. Reasonable mechanical properties might be achieved but high swelling of boards when in contact with water remains a problem (Anglès et al. 1999, 2001, Bouajila et al. 2005; Roffael et al. 2007). The lignin localised to the surface of isolated fibres offers also special possibilities for enzymes to react on. Application of redox-enzymes activating the lignin crust by formation of functional groups can enhance the natural binding forces of the lignin (Hüttermann et al. 2001, Mai et al. 2004; Fig. 3).

Different types of phenol-oxidising enzymes have been tested for their ability to activate wood lignin: various laccases, mushroom tyrosinase, and horseradish peroxidase (see Chapter 17 of this book for detailed descriptions of enzyme reactions). Laccases of e.g. *Trametes hirsuta* and *Trametes versicolor* and horseradish peroxidase efficiently oxidised and polymerised isolated lignins whilst effects of mushroom tyrosinase were neglectable (Kaplan 1979, Guerra et al. 2000, Grön-

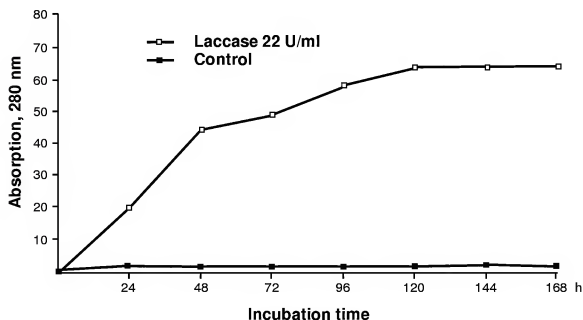


Fig. 4 Release of lignin from wood fibre surfaces by action of laccase measured in a spectrophotometer at an optical density of 280 nm (according to Kharazipour et al. 1997). In the experiment, 5 g of fibres in 500 ml buffer were treated with 22 U/ml laccase [arbitrary units used by Kharazipour et al. (1997) were converted into international units as described in Chapter 19 of this book]. After incubation with the enzyme, the buffer was 100-fold concentrated for analysis in the spectrophotometer

qvist et al. 2005, Yoshida et al. 2005). Similarly, various laccases and peroxidases are shown to attack lignin in the fibre-bound stage (Kharazipour et al. 1997, Barsberg & Thygesen 1999, Widsten et al. 2002b,c, Grönqvist et al. 2003). As an example, Fig. 4 presents that treatment of wood fibres in a suitable reaction buffer with commercial laccase from *T. versicolor* leads to a gradual release of highly oxidised lignin fragments into solution. In this type of trial experiments, the released lignin fragments had a mean molecular weight of 2 kDa at pH 5.0 and of 3.6 kDa at pH 4.5. The speed of the release of lignin from the fibre surface depended on the amount of enzyme activity in the solution. With higher enzyme activities, maximum release was achieved after two days, and with lower activities, it was after five days (Kharazipour et al. 1997, 1998b). Inspection of laccase-treated fibres by scanning electron microscopy revealed that lignin crusts on fibre surfaces were loosened after a few hours of incubation and completely removed at the end of incubation (Kharazipour & Hüttermann 1993; Fig. 3B and C).

Laccases and peroxidases both cause phenoxy radical formation when incubated with lignin (Grönqvist et al. 2005). By enzymatic treatment of wood fibres, phenoxy radicals are formed in the lignin matrix (Felby et al. 1997a,b, 1998, Barsberg & Thygesen 1999, Widsten et al. 2002b,c). These radicals contribute to the

fibre surface reactivity and may undergo radical coupling mechanism between dissolved low molecular mass phenols of lignin origin and polymeric lignin (Majtnerová & Gellerstedt 2006). Target for wood composite production is therefore not the complete enzymatic removal of the lignin from fibre surfaces but achieving optimum degrees of loosening the fibre surfaces and of lignin depolymerisation (Fig. 3B) which allow best activation of auto-adhesion forces in subsequent panel board production.

Wood composite production with enzymes

In order to replace synthetic binders, two types of enzymatic approaches have been followed up to enhance adhesion in wood composite production: i. the 1-component system where the applied oxidative enzyme serves as an activator of lignin by radical production and ii. the 2-component system where the oxidative enzyme is applied together with added lignin or lignin-like phenolics to give a two-component glue (Hüttermann et al. 2001, Felby et al. 2004, Mai et al. 2004; Fig. 5 and 6).

Enzymatic activation of auto-adhesion of wood fibres (1-component system)

In the 1-component system, the applied enzyme needs to react on the fibre surfaces. This process is applicable to MDF (medium density fibreboard) production. MDF has been made both with peroxidases and with laccases as biological catalysts (Hüttermann et al. 2001, Felby et al. 2004, Mai et al. 2004). In pressed boards, fibres were in very close contact similarly to fibres in native wood tissue. The contact zone between fibres in pressed boards contained two times more lignin than the S2-layer of the walls of the fibres (Kharazipour et al. 1998b).

First suggested in production of panel boards with enzymes (albeit for a two-component system, see below) was the use of peroxidases (Nimz et al. 1972, 1976). Kharazipour et al. (1998a) successfully followed up this idea in MDF production on laboratory scale and produced boards by incubating mixtures of spruce/pine/beech fibres in aqueous solution with a commercial Mn-peroxidase. The boards pressed after enzymatic treatment and de-watering the fibre material for 5 min at 190°C and 2.5 MPa came close to the required standards of MDF (European standard 622-5 2006). For optimum board properties, enzyme concentrations were best at a concentration of 300 U/ml with a H₂O₂ concentration of 15 mM added at intervals of 10 min over the enzyme incubation period. Optimum incubation times were 4 h. The best pH was 6.0 which corresponded to the pH optimum of the applied Mn-peroxidase. Properties depended strongly on the MDF densities. Boards had higher internal bond strength and showed lower swelling in water with increasing densities from 0.8 g cm⁻³ up to 1.0 g cm⁻³ (Kharazipour et al. 1998a, Kharazipour & Hüttermann 1998, Mai et al. 2004, Fig. 7).

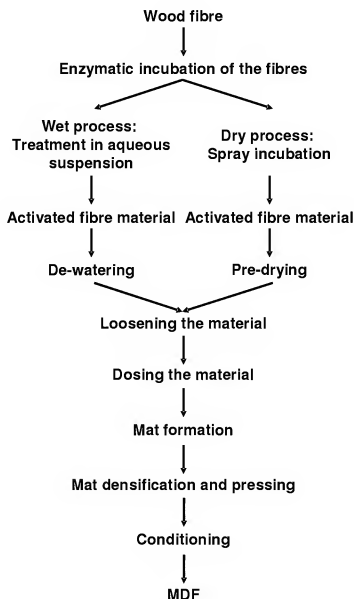


Fig. 5 The principle steps in the wet and dry treatments of wood fibres with enzymes for manufacturing medium density fibreboards (after Kharazipour 1996; figure modified from Mai et al. 2004)

Because of the high redox-potential of peroxidases, polymerisation and activation of lignin on the fibre surfaces by peroxidases might be better than that what can be achieved by laccases being enzymes with a low redox-potential (Grönqvist et al. 2005, Martínez et al. 2005). There is however the disadvantage for applications that peroxidases require H_2O_2 for enzymatic activity. Generally, handling H_2O_2 presents a safety risk due to its corrosive and explosive character. Processes for wood composite production with peroxidases were therefore never implemented on larger scale.

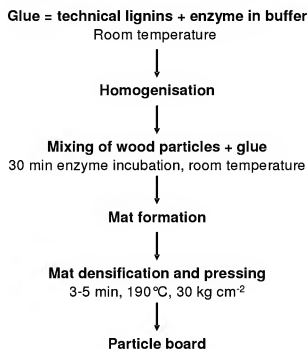


Fig. 6 The principle steps in the 2-component process for the manufacture of particle boards with technical lignins and enzymes as glue (after Haars et al. 1989 and Kharazipour 1996; figure modified from Mai et al. 2004)

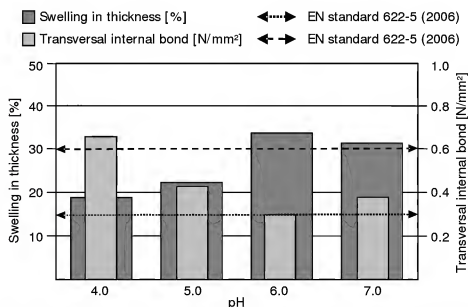


Fig. 7 Technical properties of 5 mm thick medium density fibreboards (MDF) made from fibres treated at different pH values with Mn-peroxidase and H₂O₂. Upon enzymatic treatment, boards were pressed for 3 minutes at 190 °C. Properties were tested according to European standard EN 622-5 (2006). After Kharazipour et al. 1998a and Kharazipour & Hüttermann 1998

In addition to being safe in handling, application of a biological catalyst in a technical process of wood composite production must meet several criteria before it can be implemented on industrial scale (Kharazipour & Hüttermann 1998). i. The enzyme must be produced on cheap substrates in large quantities. ii. It must permit long-term-storage after production without any loss of activity until it is required for technical use. iii. It must be applicable in crude form without prior expensive purification. iv. It must be reasonably stable at room temperature allowing necessary handling in the wood manufacturing factory. v. At best, the enzyme should be thermo-tolerant with a high temperature optimum to still allow activity during heating the fibre material. Particularly the last point laccases fulfill better than peroxidases. Peroxidases tend to react best at temperatures in the range of 30 up to 45°C (Yang et al. 2005, Rodríguez Couto et al. 2006). Many laccases in contrast are active over a much broader temperature range and react well at temperatures of 50-60°C and even above (Baldrian 2006, Kilaru 2006). Laccases (as peroxidases) can be produced in large amounts (see Chapter 19 of this book), can be applied in crude form to react on lignin and lignin-compounds (Kharazipour et al. 1997, Ortega-Clemente et al. 2004), and are often highly stable and can be stored for long periods (Baldrian 2006, Kilaru 2006, Michniewicz et al. 2006).

Laccases have therefore been more extensively studied in MDF production up to the industrial scale (Hüttermann et al. 2001, Felby et al. 2004, Mai et al. 2004). Boards produced with fibre-treated laccases are found to have higher mechanical stabilities than non-treated binder-free control boards (see e.g. Kharazipour et al. 1997, Felby et al. 1997a, 2002, Widsten 2002, Kharazipour 2002, Widsten et al. 2004). Two different production principles have been tested: dry processes and wet processes (Fig. 5). Kharazipour et al. (1997) tested mixtures of 80% spruce/pine fibres plus 20% beech fibres. Incubation of fibres with commercially available *Trametes* laccase was performed in aqueous solution (wet process) or the enzyme was sprayed onto the fibre (dry process). The type of enzyme application had no fundamental influence on MDF properties that on the whole met the effectual European standards. Best board properties (internal bond strength, swelling) were seen after an incubation of 4 h at pH 5.0 (Kharazipour et al. 1997, Kharazipour & Hüttermann 1998). Felby et al. (1997b, 1998, 2002) tested thermo-mechanical pulp of beech in a wet and in a dry 1-component system of a different set-up, either with commercial *Trametes* laccase or with a thermotolerant laccase from the ascomycete *Myceliophthora thermophila*. Incubation times (30-60 min), concentrations of *Trametes* laccase (3.5 to 24 units/g fibre), and the pH of the solution (pH 4.5) applied by Felby et al. (1997b, 1998) were lower than in the studies by Kharazipour and colleagues but resulting boards had comparable bond strengths (Mai et al. 2004). Board thickness swelling after 24 h in water was considerably high for the boards made by the wet process (57% thickness swelling of 3 mm thick MDF made at a density of 1.037g/cm³) whilst in the dry process a step of forced-air-drying drastically reduced the swelling capacity of produced MDF (19%

thickness swelling of 3 mm thick MDF; Felby et al. 1997a) to values much below the permitted limit (27% thickness swelling of MDF of such density and width; European standard EN 662-5 2006).

Boards made in a dry process in pilot scale with laccase from *M. thermophila* had comparable strength properties to conventional UF-bonded boards. However, the long pressing times (30 min) and lower moisture resistance of enzyme-bonded boards require adaptations to the process to make it economically feasible in practice. Adding wax as a hydrophobiser interfered with proper gluing and reduced the strength properties of boards (Felby et al. 2002).

Electron-microscopic views of enzyme-bonded fibres show smooth continuous lignin crusts formed across the fibres. This suggests that lignin on the individual fibre surfaces melted during pressing to subsequently set into such continuous films during cooling down. Sealing was stronger in enzyme-treated samples from a dry process than in samples from a wet process whilst conventionally bonded boards showed only punctiform interconnections (Kharazipour 1996; Fig. 8). Certainly it can be deduced from these studies, that enzymatic treatment altered the surface properties of the fibres. Chemically, the individual alterations on the material might however not as easily be determinable in the lignocellulosic bulk material even not when using sensitive techniques such as FTIR (Fourier transform infrared) spectroscopy (Schnidt et al. 2002).

Felby & Hassingboe (1996) assumed that colloidal lignin acts as a charge mediator between laccase and lignin on the fibre surface. Their research group followed up the generation of phenoxy radicals by *Trametes* laccase in beech fibre suspensions. The concentration of phenoxy radicals in enzyme-treated suspensions was 5 to 6 times higher than in parallel non-treated samples and kept stable for at least two weeks (Felby et al. 1997b, 1998). Next to a change in the chemical composition of the surface, laccase-treated fibres showed a markedly increase in hydrophobicity. Felby et al. (2004) deduced from these observations that lignin extractives were precipitated on the fibre surface. These surface-deposited lignin extractives might serve in covalent fibre bonding by promoting surface molecular entanglements and through radical-mediated cross-linking (Felby et al. 2004).

An increase of radicals in fibres and similar stabilities of generated radicals at room temperature was also observed when treating fibres of other hardwood species (aspen, birch, eucalypt) and softwood (spruce, pine) with laccase (Widsten 2002, Widsten et al. 2002b,c, Lund et al. 2003). The temperature used for defibration fibres had an influence on amounts of low-molecular weight lignin as a proposed substrate for laccase. Consequently, more radicals formed by laccase on fibres from pulps of higher defibration temperatures and higher amounts of radicals positively influenced MDF properties (Widsten 2002, Widsten et al. 2001, 2002a,b,c, 2003, 2004).

Since amounts of laccase-generated radicals clearly influence board properties (Widsten 2002, Widsten et al. 2003), an obvious strategy to improve the MDF

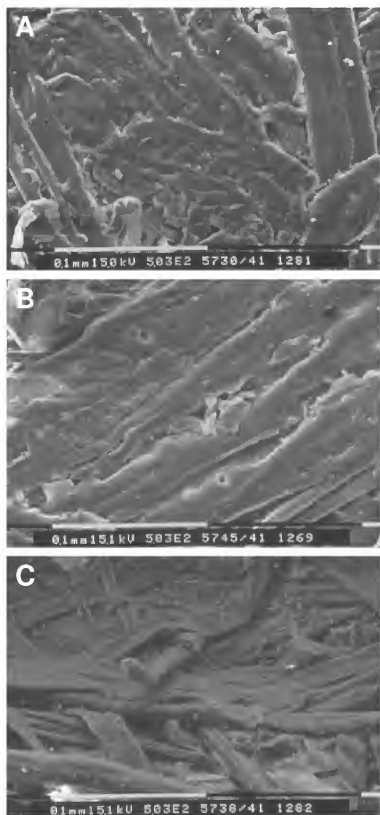
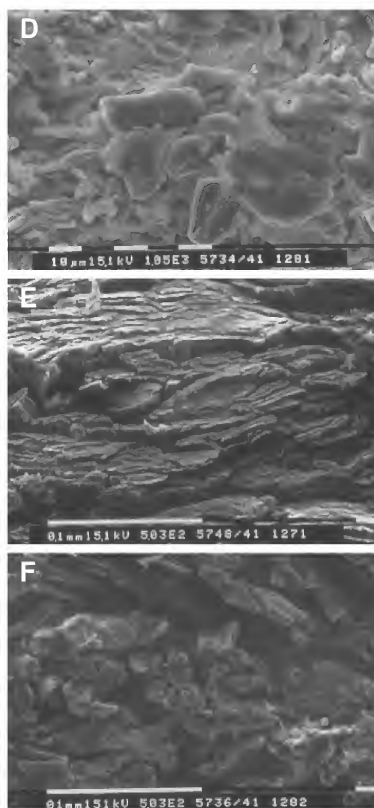


Fig. 8 Scanning electron microscope (SEM) pictures of fibreboards produced from blends of spruce, pine, and beech fibres with *Trametes* laccase in a wet production process (A. cross and D. tangential section), with laccase by spray incubation in a dry



production process (B. cross and E. tangential section), and with conventional resin (C. cross and F. tangential section). After Kharazipour (1996)

production process is to optimise conditions for radical production. The time of enzyme incubation must be long enough to activate the lignin but not too long to ensure that the lignin still remains on the surface of the fibres. Using the optimal pH of the enzyme ensures highest enzyme activity (Kharazipour & Hüttermann 1998). Nevertheless, currently applied incubation times of 30 and more minutes are too long to be economical. An important hindrance in reaction efficiency is its low redox-potential of the enzymes (Solomon et al. 1992, Shleev et al. 2004, Baldrian 2006, Kilaru 2006). Screening for mutations by modern molecular biological techniques can yield enzymes with higher redox-potential for recombinant production (Alcalde et al. 2006, Zumarraga et al. 2007; further reading in Chapter 19 of this book). Currently, *M. thermophila* laccase mutants with a 3-fold increase in activity are available (Alcalde et al. 2006) but this is still not satisfying enough for economical MDF production.

Laccases need an idle phenolic group at the aromatic ring for oxidation. This circumstance hinders the application of laccase in lignin-biotechnology since in natural lignin most of the phenolic groups are substituted (Leonowicz et al. 2001, Rochefort et al. 2004). Luckily, the detection of laccase mediators, small organic molecules with a high redox-potential (Bourbonnais & Paice 1990, Eggert et al. 2006, Johannes et al. 1996, Majcherczyk et al. 1998, 1999), opened up chemical solutions to overcome weak properties of laccases (Call & Mücke 1997). Application of mediators enhances laccase reaction speeds and, most importantly, the substrate spectrum of the enzymes broadens dramatically. Mediators are natural or artificial substrates to the enzyme and operate as redox-molecules between laccases and other compounds, regardless of whether these are a direct (natural or artificial) substrate of the enzymes or whether these are not a substrate to the enzymes. When oxidised by laccases, mediators can oxidise non-enzymatically other phenolic or non-phenolic compounds (including non-phenolic lignin substrates) by transfer of electrons with ionisation potentials exceeding the potentials of laccases. Without being degraded, true redox-mediators perform many reaction circles between the enzymes and other compounds (Call & Mücke 1997, Johannes & Majcherczyk 2000, Morozova et al. 2007; see Fig. 1 and accompanying text in Chapter 17 of this book). Most compounds known to enhance laccase enzymatic actions are eliminated from the reaction by chemical secondary transformations after one or a few cycles and might thus rather be called “enhancers” (Morozova et al. 2007). Currently, few compounds have been described that perform considerable numbers of redox cycles (Bourbonnais et al. 1998, 2000, Johannes & Majcherczyk 2000, Fabbrini et al. 2002). There is thus an active screening on-going to identify non-toxic, inexpensive and highly efficient substances acting as true mediators (Rochefort et al. 2004, Morozova et al. 2007). Nevertheless, first functional laccase-mediator systems have been developed for the use in different industries – for fabric bleaching, cork modification, and, last but not least, for paper pulp delignification where complete delignification is wanted (Call & Mücke 1997, Rodri-

guez Couto & Toca Herrera 2006, Morozova et al. 2007). ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulphonic acid) has been shown to mediate electron abstraction between fibre lignin and laccase in qualitative difference in temporal phases than laccase does alone. Resulting distinguished lignin modifications may have consequences for subsequent fibre behaviour (Barsberg 2002). Lund & Felby (2001) applied a range of mediators in laccase-modification of spruce and pine fibres and found ABTS and PPT (phenothiazine-10-propionic acid) to be effective in enhancing wet tensile strength of paper handsheets made from the pulp. Similar results were obtained with ABTS by Elegir et al. (2005, 2007). Treatment of fibres with laccase and gallic acid were also reported to change wet tensile strength (Chandra et al. 2004b), indicating that laccase-mediator system can influence properties of the fibre surfaces. Various mediators are therefore currently tested in MDF production with laccase in order to achieve higher reactivity between laccase and lignin and better board properties (Müller et al. 2006). The unfortunately limited availability of cheap and non-toxic mediators is of course a restricting factor in such approaches (Rochefort et al. 2004, Morozova et al. 2007). So far, in laccase-mediated bleaching of lignocellulosic fibres 1-hydroxybenzotriazole and the lignin-derived phenols acetosyringone and syringaldehyde acid have been positively tested as mediators whereas negligible effects were seen with *p*-coumaric acid (Ibarra et al. 2006, Camamero et al. 2007, Gutiérrez et al. 2007). Oppositional ABTS-mediated depolymerisation and polymerisation effects on kraft lignin samples (González Arzola et al. 2006, Hernández Feraud et al. 2006) imply that the doses and times of mediators and enzymes applied have to be expected to play an important role in activating of fibre material. In line with this, dosages of 4-hydroxybenzoic acid were most influential in laccase-mediated modification of paper pulp (Chandra et al. 2004a).

Further to applications of mediators to improve mechanical properties by laccase action, recent studies suggest that a successive xylanase and laccase treatment with or without mediators might also be useful in efficiently modifying fibre surfaces at least in paper production. As the gallic acid mediator treatment, successive xylanase-laccase treatment has been reported to increase the wet tensile strength of paper (Chandra & Raugaskas 2005, Kapoor et al. 2007).

2-component adhesives based on lignin and enzymes

In the wood composite industry, addition of lignosulphonates (spent sulphite liquor) or kraft lignins from sulphate pulping, to conventional PF resins is targeted at replacing parts of the phenol component made from petrochemicals (e.g. see Roffael & Rauch 1971, Shen 1974, Nimz 1983, Ayla & Nimz 1984, Danielson & Simonson 1998a,b, Tejado et al. 2007; further reading in Chapter 16 of this book). Formaldehyde from the PF resin may act in such mixtures as a cross-linker between the reactive sites of the technical lignins and the synthetic phenol binder (Kuo et al. 1991, Vázquez et al. 1999, El Mansouri & Salvadó 2006, 2007). As first

suggested by Nimz et al. (1972, 1976), radical polymerisation of the technical lignins (lignosulphonate) set off by oxidative enzymes is an alternative way of cross-linking (Kharazipour et al. 1998b, Hüttermann et al. 2000, 2001).

Haars et al. (1989) first described a 2-component process for particleboard production with a mixture of spray-dried sulphite liquor and concentrated culture filtrate of *T. versicolor* containing laccase activity (crude enzyme), following the principle scheme presented in Fig. 6. Whilst pressed boards with 0.42–0.47 N/m² fulfilled well the requirements for transverse tensile strength (European standard EN 312-3 2003), they failed in dimensional stability according to European standard EN 312-4 (2003; 24 h swelling in water) due to the hydrophilic nature of the sulphonate groups present in the soluble lignosulphonate.

Long ago, laccases had already been demonstrated to polymerise lignin, first *in vivo* (Hüttermann et al. 1977) and then *in vitro* in buffers in reaction tubes (Haars & Hüttermann 1980a,b). The typical enzyme reaction of the soluble laccases takes place in water with, in principle, a hydrophilic substrate. The reaction product, however, must be water-insoluble and hydrophobic in order to meet technical requirements in particle board production. Changing in the reaction the water-soluble lignosulphonates to mainly water-insoluble lignins (kraft lignin) with addition of small amounts of conventional resins helped to overcome the swelling-in-water problem. Swelling of boards was reduced when adding one part of methylene diphenyl diisocyanate (PMDI) binder to nine parts of laccase-modified kraft lignin binder. Swelling of particle boards was halved and tensile strength doubled compared with individual treatments of either lignin-laccase or PMDI, respectively. As further positive effect of usage of laccase-modified kraft lignin, resulting boards were completely emission free (Kharazipour et al. 1991, Kharazipour 1996, Hüttermann & Kharazipour 1996, Hüttermann et al. 2001). The current state of art might however be further improved through application of mediators. Enzyme-mediated polymerisation reactions of kraft lignins have been shown to be much enhanced by addition of mediators such as ABTS or hydroquinone (González Arzola et al. 2006, Shilev et al. 2006).

A 2-component system has also been tested in paperboard manufacturing. Yamaguchi et al. (1991, 1992) applied dehydrogenative polymerisation of vanillin acid, mimosa tannin, and tannic acids and either crude peroxidase from madeke bamboo or *T. versicolor* laccase to thermo-mechanical pulp. Upon lap-joining, pressing, and drying of sheets obtained from such treated pulp, an increase in tensile and ply-bond strength was observed in resulting paperboard. The underlying mechanism for the increase in mechanical properties is thought to be caused by loosening the macromolecular lignin structure on the surface of the fibres and by copolymerisation of the new lignin with the residual lignin on the surface of the pulp (Yamaguchi et al. 1994, Hüttermann et al. 2001). In kraft pulps, the lignin content on the fibre surface could be two to three times higher than the bulk lignin concentration and about 30% of the surface might consist of lignin (Sjöberg et

al. 2002). In CTMP (chemi-thermo-mechanical pulp), the surface lignin is estimated 50% (Westermarck et al. 1988). Since laccases themselves can not penetrate the fibre wall and into the lignin, Elegir et al. (2007) predict that a total of 6% of the kraft pulp surface and a total of 10% of the CTMP surface, respectively, are directly accessible to laccase action. These authors added laccase from *Trametes pubescens* together with ultra-filtered lignin (UFL) to these types of pulps and found impressive two-fold increases in wet tensile strength of paper handsheets compared to no laccase or only laccase treatments. Handsheets made from laccase/UFL-treated kraft pulp retained other critical mechanical properties (dry tensile strength, compression strength, and Scott Band internal strength) unlike handsheets from laccase/ABTS treated kraft pulp. These latter handsheets had however an even better wet tensile strength than those from the laccase/UFL pulp.

Brown-rotted lignin obtained by fungal decay from wood (Goodell 2003) displays a stronger reactivity with formaldehyde than technical lignins such as kraft lignin. Brown-rot fungi modify lignin through demethylation. Brown-rotted lignin can therefore easily replace up to 35% of PF resins in panel board production (Jin et al. 1990a,b). Brown-rotted lignin was therefore also tested in a 2-component system with horseradish peroxidases, respectively fungal laccase for bonding wood laminates. Shear strength of laminates were low and the samples were not water proof (Jin et al. 1991). Such system will unlikely be ever feasible in practice, also because brown-rotted lignin is hard to get (Mai et al. 2004).

Fungal fermentation of wood for the wood panel industry

Removal of lignin, especially that in the middle lamellae as the natural glue between wood fibres, is a key step for fibre production (Johansson et al. 1999; Fig. 2). In common thermo-mechanical pulping, the middle lamellae lignin is plasticized at temperatures above its glass-transition point and this leads to defibration (see above and Chapter 15 of this book). Generally, fibre production by thermo-mechanical treatment of wood chips at high pressure, high temperatures, and strong mechanical sheering forces is highly energy demanding. Energy consumption for fibre separation is thus the major cost factor to be considered when calculating production costs for paper and for wood fibre composites (Sabourin 2000, Browne et al. 2006). Incubation of wood chips in solid-state fermentation (SSF; for detailed explanations see Chapter 19 of this book) with either white- or brown-rot fungi drastically reduces the energy required for defibration. The energy demand for pulping could be reduced by such treatments by factors of up to 40%. Furthermore, due to the enzymatic and chemically-induced actions performed by the fungi on the cell walls of the fibres, there was also a decreased demand for petrochemical resins (up to 35%) when pressing the fibres from fungal-incubated wood chips into boards. MDF pressed from such fungal-treated wood chips had

excellent technical properties (Wagenführ 1998, Körner 1990, Körner 1993, Kühne 1993, Unbehaun et al. 2000).

Fackler et al. (2007) analysed the surface of *Ceriporiopsis subvermispora*-treated wood shavings. Already three days after inoculation with the white-rot fungus, at the time of onset of enzyme-production, radicals were formed in the wood and delignification became significant (Schwanninger et al. 2004, Fackler et al. 2007). After about one week to 10 days of incubation, the total lignin content decreased by 3.5% and extractable components were formed (Guerra et al. 2004, Fackler et al. 2006, 2007). Small scale gluing tests of white-rot modified spruce veneer strips with aminoplastic resins were performed. Shearing forces that could be applied to the veneers were by 16-21% higher compared to glued untreated strips (Fackler et al. 2007). Another recent work states that incubation of aspen strands with the white-rot fungus *T. birsutus* gave a 15% reduction in adhesive consumption for OSB production accompanied by an increase in internal bond strength (Yang et al. 2007).

The work by Fackler et al. (2007) shows that already shortly incubated wood shavings were positively affected by *C. subvermispora* and other fungi in their properties for usages in wood composite production. For wood fibre production of larger amounts of wood chips more time for fungal incubation is however needed. The process of wood fermentation by fungi in SSF is usually time-consuming and can take a few weeks to months and is in addition prone to infections (Wienhaus et al. 1978, Wagenführ 1988, Körner 1990, Körner 1993, Körner et al. 1993, 2001, Unbehaun et al. 2000). Taking instead partially degraded wood directly from forests with large capacities of suitable decaying trees saves fermentation costs and time and also storage place. This approach is followed up in one of our current research projects as described below. In consequence of this work, an abundant biological product from conifer forests - which so far is considered to be waste and little or non-marketable at all - will strongly increase in value.

***Heterobasidion* infested wood - naturally activated fibres as a raw material for the wood panel industry**

Sizeable stocks of naturally fermented wood are available in Germany and other European countries with temperate and boreal climates in conifer forests where *Heterobasidion annosum sensu stricto* (formerly P-type intersterility group) and relatives occur. These basidiomycetes are major pathogens to conifers (Asiegbu et al. 2005; see also Chapters 14 and 24 of this book). To infect potential host trees, spores of these fungi need to drop and germinate either on freshly cut stumps or on wounds at roots and stems of the conifers (Vasiliauskas et al. 1996, 2002, Vasiliauskas & Stenlid 1998b, Rönnberg 2000, Vasiliauskas 2001; Fig. 9). Spores are prevalent in high densities in the air above conifer forests. Maximum spore deposition rates of 1,150 spores per m² and h have been determined (Gonthier et al. 2001, 2005). In



Fig. 9 Sources of primary *Heterobasidion* infections in the forests: infection via stumps (left), damages caused by deer (middle), and damages caused by yarding (right)

cidences of infections via spore germination are therefore numerous, particularly after summer thinnings (Rönnerberg et al. 2006). From primary infections, *Heterobasidion* can spread vegetatively via root contacts to neighboured healthy trees (Piri 1996, Vollbrecht & Stenlid 1999). A further infection source are wood pieces within soils left from clear-cuttings (Piri 2003, Redfern & MacAskill 2003). In consequence, in older conifer forests up to 100% of all trees might be infected by *Heterobasidion* (Rönnerberg & Jørgensen 2000; for detailed data on infection frequencies in different conifers and different countries, see Chapter 24 of this book).

Right at the beginning, an infestation is not visible on the tree. Once *Heterobasidion* entered a standing tree, it will grow from infected roots or bark wounds upwards into the heartwood of the stem. The fungus grows in a pyramidal manner from the bottom of the trunk to the top. In this way with time, the interior of the tree becomes rotten and the stem loses its mechanical stability. Average decay columns of later stages of infestation within the trees measure in between 3.3 to 4.6 m (Vasiliaskas & Stenlid 1998a; Fig. 10). However, decay columns as long as 12 m have also been observed (Stenlid & Wästerlund 1986). In an advanced stage of infestation, some trunks have a bulged appearance by anatomical defence responses, resin exudation might occur, crown deterioration is often observed, and fruiting bodies might form on the surface of roots and the base of stems (Greig 1998, Krekling et al. 2004, Omdal et al. 2004). If any of those symptoms are visible, one can be sure that the fungus has already reached an internal decay dimension as shown in Fig. 10. In nature, such decay in standing stems is an ecologically necessary process, since it finally leads to collapse of the stems and formation of gaps in the stands in which natural regeneration can start (Slaughter & Parmeter 1995, Rizzo et al. 2000, Rizzo & Slaughter 2001, Bendel et al. 2006a,b; see also Fig. 2 in Chapter 24 of this book). From the economic point of view, *Heterobasi*



Fig. 10 Development of the fungus within the trunk. The lower 3 m of a felled stem of spruce have been trimmed due to an advanced decay by *Heterobasidion*. With the exception of the small outer sapwood ring, all interior wood is light-brownish stained due to the fungal infestation. By the advanced progress of decay, the outer sapwood was easily lifted from the decay column of the inner heartwood

dion is a threatening disease because of the loss of marketing quality of the infected stems. Reductions in value of infested wood are high: 10% of cut stems are calculated to be lost yearly alone in Europe (Delatour 1980), whilst in single stands, losses in final cuttings and reductions in sales revenues can be much higher (Tamminen 1985, Kaarna-Vuorinen 2000; further discussion in Chapter 24 of this book).

Heterobasidion species are white-rot fungi (Asiegbu et al. 2005; see also Chapter 17 of this book). Laccase activities in *Heterobasidion* have been described (Haars & Hüttermann 1980a,b, 1983, Daniel et al. 1998, Johansson et al. 1998, Al-Adhami et al. 2002, Asiegbu et al. 2004, Lang 2004) and genes for Mn-dependent peroxidases are known to exist in the genus (Majjala et al. 2003). Laccases play probably a major role in the lignin degradation of wood, but little detail is known about such fungal enzymatic activities on lignin (further reading in Chapter 17 of

this book). Particularly *H. annosum* is very aggressive towards wood and also very active in laccase production (Haars & Hüttermann 1983, Daniel et al. 1998, Lang 2004). Furthermore, *Heterobasidion* isolates secrete several hemicellulolytic enzymes (xylanases, mannanases, α -galactosidases) and endoglucanases (Majjala et al. 1995) in presence of wood. Pectinase activities have been described (Johansson 1988, Karlsson & Stenlid 1991), and genes for cytochrome P450 monooxygenases were shown to be transcribed at the stage of spore germination (Abu et al. 2004). Whether all these enzymes are indeed necessary for wood degradation, and how they possibly interact on spatial and time scales is currently unknown. In any case, the fungal-infected wood is biologically modified since white-rot fungi such as *Heterobasidion* attack lignin (see Chapter 17 in this book). For economic and ecological reasons, this is potentially advantageous for the industrial production of wood composites and gives seemingly inferior timbers an added-value.

Firstly, the expensive, energy consuming steps necessary for particle and fibre production from wood can be expected to decrease. Crushing *Heterobasidion*-infested wood into chips is comparably easy to perform by the loose overall structure of the material (see Fig. 11 and in Chapter 24 of this book Fig. 4 and the accompanying text). Fibre production is technically more demanding by the fact that the wood chips need to be thermo-mechanically treated at high pressure, high temperatures and strong mechanical sheering forces (see above and Chapter 15 of this book). However, also here, by the already loosened fibre structure costs for energy can be saved (Körner et al. 2001).

Secondly, by the enzymatic activation of the fibre lignin during the fungal decay, amounts of synthetic binders can be reduced in wood composite production. With fibres resulting from forced fermentations with white- and brown-

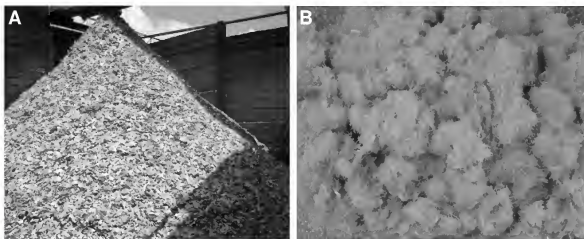


Fig. 11 A. Heap of wood chips obtained from *Heterobasidion*-infested spruce (see also Fig. 4 in Chapter 24 of this book). B. Fibres from thermo-mechanical pulping of wood chips from *Heterobasidion*-infested spruce

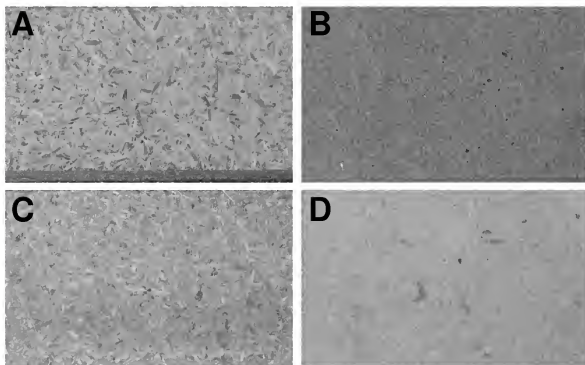


Fig. 12 Particle- and fibreboards made from conventional industrial wood chips (A, B) and from *Heterobasidion*-infested spruce (C, D). Note the lighter colours of the boards made from *Heterobasidion*-infested spruce

rot fungi, savings in form of up to 35% reduction in adhesives was shown to be possible in MDF production without affecting the quality of the final products (Wienhaus et al. 1978, Wagenführ 1988, Körner et al. 1993, 2001). The same is expected to hold true for fibres from natural biological wood modification taking place in the forests by *Heterobasidion*.

Furthermore, there are other advantages to the *Heterobasidion*-infested wood. Particle- and fibreboards produced by the infested wood have a brighter, more attractive colour than boards from standard industrial spruce wood chips and fibres (Fig. 12). Small test scales revealed that in comparison to healthy wood, the biologically transformed wood has a lower gross density by 30% (C. Bohn et al. unpublished). Under a specific process management, this will enable production of new board types of low weight that for example can find application in insulation of ceilings.

A laboratory test of *Heterobasidion*-infested spruce wood in the production of wood composites

For first trials of testing the suitability of *Heterobasidion*-infested wood for the production of particle- and fibreboards, infested spruce wood was chipped and defibrated by conventional techniques (Fig. 11). Three-layered particle boards and

MDF were produced by conventional manufacturing technology under addition of urea-formaldehyde (UF) resin and a hydrophobic substance (paraffin wax), and properties of the boards were compared to those of boards produced in parallel from non-infested spruce wood (Tables 1 and 2).

The 19 mm-thick three-layered particle boards produced from *Heterobasidion*-infested spruce had internal bond strengths between 0.54 and 0.88 N/mm² (Table 1). The minimum required value specified by European standard EN 312-3 (2003) is 0.35 N/mm² and the boards clearly exceeded this value. According to Heller (1995), a reduction in density by 100 kg/m³ goes along with a decrease in the internal bond strength by about 0.2 N/mm². Such a relationship was also detected in our experiments. The internal bond strength declined by about 0.17 N/mm² per 100 kg/m³ reduction in density (compare Table 1). Nevertheless, even at the lowest tested density (500 kg/m³), the strength of the boards were better than defined in the standard.

For applications of particle boards such as usage in the furniture industry, the stability of the surface is as important as the internal bond strength (Deppe & Ernst 2000). Also the surface strengths of particle boards made from *Heterobasidion*-infested spruce outranged the minimum value of 0.80 N/mm² defined in European standard EN 312-3 (2003) and, in all instances, the values were better than those of the reference boards made from non-infested spruce (Table 1).

Table 1 Properties of three-layered test particle boards (19 mm thickness) made with UF resin from non-infested and from *Heterobasidion*-infested spruce

Property*	Type of spruce wood	Density (kg/m ³)				
		500	550	600	650	700
Internal bond strength (N/mm ²)	Non-infested	0.40	0.51	0.65	0.72	0.75
	<i>Heterobasidion</i> -infested	0.54	0.61	0.67	0.80	0.88
Surface strength (N/mm ²)	Non-infested	0.82	0.89	1.23	1.39	1.56
	<i>Heterobasidion</i> -infested	0.95	1.05	1.30	1.58	1.64
Swelling (%) after 2 h in water	Non-infested	5.06	4.91	4.15	4.09	3.94
	<i>Heterobasidion</i> -infested	4.66	3.85	3.90	2.96	2.68
Swelling (%) after 24 h in water	Non-infested	12.9	14.8	13.9	15.2	15.7
	<i>Heterobasidion</i> -infested	13.9	13.9	14.4	12.3	12.4

* Internal bond strength was tested according to European standard EN 319 (1993), surface strength according to European standard EN 311 (2002), and swelling after 24 h in water according to European standard EN 317 (2003)

The maximum allowed value for swelling of particle boards of 19 mm width after 24 h in water is 15% (European standard EN 312-4 2003). Through application of a hydrophobic substance on paraffin wax basis, swelling of particle boards from *Heterobasidion*-infested spruce was below this threshold (Table 1). Compared to reference boards from non-infested spruce, the colour of the surfaces of *Heterobasidion*-infested wood were brighter (Fig. 12) and this is an advantage for surface coating with fine melamine paper for lamination (Neusser 1979, Deppe & Ernst 2000).

Regarding the MDF made from the fungal modified wood under addition of 12% MUF (melamine-urea-formaldehyde) resin, the internal bond strength with rising density ranged from 0.43 to 1.36 N/mm² (Table 2). There was an increase in internal bond strength by approximately 0.31 N/mm per 100 kg/m³ increase in density. At a density of 600 kg/m³ and above, all the 16 mm-thick boards exceeded the requested minimum internal bond strength of 0.55 N/mm² (European standard EN 622-5 2006). The minimum bending strength allowed for 16 mm MDF by European standard EN 310 (1993) is 20 N/mm². Even with the lowest density applied (500 kg fibre/m³), this value were reached and with increasing densities it was surpassed (Table 2). Also in MDF production, addition of a paraffin wax-based substance gave rise to boards with swelling values below 12% as requested by European standard EN 622-5 (2006). Altogether, the presented observations imply that it should be possible to reduce the load of the bonding agent in wood composite production from *Heterobasidion*-infested spruce.

Conclusions

Alone in Germany, 13 million m³ particle and fibreboards are every year produced (VHI 2007; see Chapter 15 of this book). This presents a huge potential market for biologically transformed wood in production of wood composites if these provide technical characteristics in the same quality as conventional boards. Usage of wood particles and fibres biologically transformed prior or during the process

Table 2 Properties of medium-density fibreboards (16 mm thickness) made from *Heterobasidion* infested spruce under addition of 12% MUF resin

Property*	Density (kg/m ³)			
	500	600	700	800
Internal bond strength (N/mm ²)	0.43	0.59	0.86	1.36
Bending strength	20	24	42	45
Swelling (%) after 24 h in water	12.4	10.1	8.9	6.3

* Internal bond strength was tested according to European standard EN 319 (1993), bending strength according to European standard 310 (1993), and swelling in water according to European standard EN 317 (2003).

of board production will reduce the load of toxic chemicals (particularly formaldehyde) that are conventionally used as bonding material. Boards resulting from ecologically friendly production can contribute to the general human well being when used in building construction and for furniture by avoiding one possible cause for the sick building syndrome (SBS; see Chapters 11 and 12 for further explanations).

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19. Production of Laccase and Other Enzymes for the Wood Industry

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Introduction

Enzymes of importance for the wood and the paper and pulp industries (see Chapter 17 of this book), in many instances, are won from fungi, either naturally or recombinantly produced. For natural enzyme production, fungal strains are selected that are most efficient in secretion of enzymes with required characteristics. Growth parameters and regulation of protein production need to be defined to establish the best conditions in the production processes, either in fermenters with a liquid medium (**submerged fermentation, SmF**) or in **solid state fermentation (SSF)** on compact moist substrates or on an inert matrix (Persson et al. 1991, Haltrich et al. 1996, Hölker et al. 2004, Hölker & Lenz 2005). Regardless of type

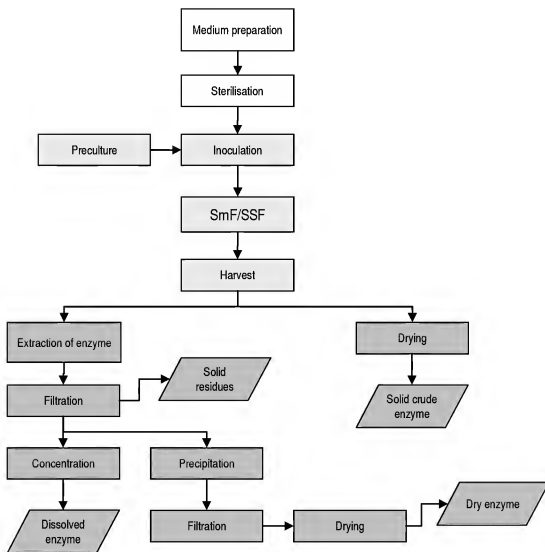


Fig. 1 General flowchart of enzyme production: steps in upstream-processing are shown in white boxes, steps of fermentation in light grey boxes, and steps in downstream-processing in darker grey boxes (adapted from Cen & Xia 1999)

of fermentation, the whole process is subdivided into three basic steps: i. upstream-processing which includes preparation of the growth substrate (most often in sterile form), ii. the fermentation process itself starting with inoculation of the production organism(s) and finishing with the harvest of the product (here enzymes), and iii. downstream-processing of the product that might be enriched and purified from the medium and production organisms and subsequently either recovered in a liquid or dried (Fig. 1).

In cases where natural strains do not yield enough products, mutagenesis and conventional genetics with appropriate screening and selection of best strains

might help to significantly improve the production rates (Parekh et al. 2000, Adrio & Demain 2006, Zhang, Y.H.P. et al. 2006). For every newly developed strain, there is further opportunity to raise the titers of products by medium modifications (Lee et al. 2005, Demain 2006). As a transgenic approach, genes for enzymes of interest are expressed under control of highly efficient promoters in homologous or in heterologous hosts, i.e., either in the species the genes came from or in a foreign species (Punt et al. 2002, Kilaru et al. 2006b). This molecular biological approach offers in addition the possibility to genetically manipulate genes in order to optimise the properties of their products to required needs (Cherry & Fidantsef 2003). Such molecular approaches, however, have also their own problems. Highly efficient promoters and an efficient gene transfer system are needed (Kilaru et al. 2006b), and problems in protein misfolding and aggregation, protein secretion, faulty protein modifications, and foreign product degradation have to be overcome (Gerngross 2004, Sørensen & Mortensen 2005, Wang et al. 2005, Guillemette et al. 2007).

In order to explain basic principles used to establish efficient enzyme production systems, we focus in this chapter on production of fungal cellulases, xylanases and, more intensively, laccases as enzymes acting on lignocellulose. However, at places, bacterial enzymes will also be incorporated. Additional aspects on production processes and regulation of expression and product degradation are discussed by Pandey et al. (1999), Beg et al. (2001), Conesa et al. (2002), Bai et al. (2003), Mach & Zeilinger (2003), Bergquist et al. (2004), Ikehata et al. (2004), Hölker et al. (2004), Aro et al. (2005), Nevalainen et al. (2005), Polizeli et al. (2005), Schügerl (2005), Sukumaran et al. (2005), Wang et al. (2005), Favela-Torres et al. (2006), Rahardjo et al. (2006), and Viniegra-Gonzalez & Favela-Torres (2006). Several of these review papers concentrate also on enzymes acting in lignocellulose degradation.

Submerged fermentation (SmF)

At the present time in Western countries, industrial production of enzymes by micro-organisms is nearly exclusively (90%) conducted in submerged fermentations in liquid media (Hölker et al. 2004). The operation of enzyme production takes place in a specific vessel, known as **fermenter** or **bioreactor**, in which micro-organisms are cultured with suitable substrates under defined process parameters. In liquid cultures, a wide variety of fermentation systems are available that distinguish by the way of oxygen input into the liquid substrate. Three main types of fermenters are available (see Fig. 2A): i. **stirred vessels** in which the liquid and the gaseous phase are mixed by stirrers, ii. **loop fermenters** in which the medium is pumped through an external circuit, and iii. **bubble columns** or **airlift reactors** without movable mechanical elements in which gas is supplied from outside and where air bubbles help in mixing (Russell et al. 1974, Schügerl 1980, Crueger &

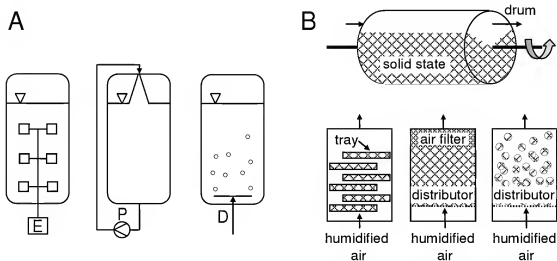


Fig. 2 Schemes of different bioreactor types. A. Submerged cultivation (SmF), from left to right: stirred vessel with engine (E) turning stirring blades, loop reactor with a pump (P) circulating the medium, and a bubble column with a gas dispenser (D). Note, the triangles mark the surface of the liquid medium. Modified from Crueger & Crueger (1984). B. Solid state fermentation (SSF): rotating drum reactor allowing mixing of the substrate (top), tray fermenter with sprinkling and circulating humidified air for humidifying the substrate on the trays (left), static column reactor or fixed-/packed-bed reactor where the solid substrate is retained on a perforated base (distributor) through which humidified air is passed through and lead into the solid substrate and an air filter to trap any dust and biological material evaporating with the air flow (middle), and fluidised bed reactor (right) used to force humidified air through a distributor into the vessel at a velocity supporting pneumatic agitation of the solid substrate particles. In each case, the solid substrate (respectively inert matrix) is indicated by the plaid pattern. Schemes were simplified from diagrams by Rodríguez Couto & Sanromán (2006b). Further explanations in Table 1

Crueger 1984, Merchuk 2003). The probably most often used reactor is the stirred vessel, both in laboratories as well as in industrial applications (Fig. 3A).

Next to classification of bioreactors, the process itself can be categorised into **batch**, **fed-batch** and **continuous cultivation**. In laboratories, enzyme production processes are usually performed in batch cultures, whereas in pilot plants and industrial applications a continuous cultivation is preferred as a complex, but more reliable and 'cheaper' cultivation technique. Batch and fed-batch operations are discontinuous fermentation processes where the culture together with the products is harvested at the end of the process (Longobardi 1994). In a batch operation, the volume of the medium is constant during the whole period; no additional nutrients are added. The fed-batch cultivation is similar to the batch cultivation

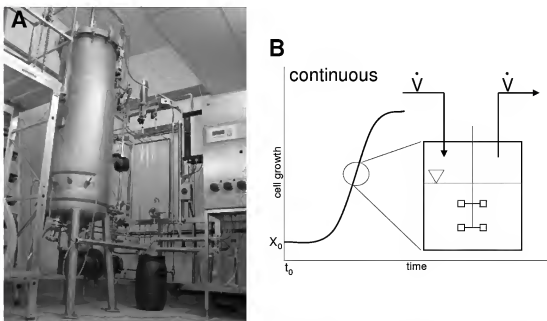


Fig. 3 500 l stirred vessel fermenter at the Institute of Forest Botany, Georg-August-University Göttingen (A) and scheme of a stirred tank reactor (compare Fig. 2A) at balanced operation (B): once after start of the process the biomass has reached an ideal density in the exponential growth phase, the inlet and the outlet volume flow of medium (indicated by the \dot{V} symbols with dot) are levelled which allows continuous cultivation

until substrate limitation is reached but it offers the possibility to control the substrate concentration at a desired condition. Once the substrate is used up in fed-batch cultivation, extra substrate is added at a specific volume flow until the maximum working volume of the bioreactor is reached. The subsequent harvest of the product and/or the micro-organisms marks the end of the cultivation.

Integrated bioprocessing in which a potentially inhibitory product (and also possible toxic by-products) is continuously removed from the fermentation broth as it is produced has important advantages in improving yield and conversion relative to conventional batch and fed-batch processes. In continuous production, product yields are enhanced by both, an absolute increased product formation and an avoidance of product decomposition (Schügerl 2000, Schügerl & Hubbuch 2005). For continuous fermentation, a steady state of the process must be achieved. At this specific cultivation stage, the substrate and biomass concentration is maintained constant by simultaneously draining of culture liquid (including the product and any by-products) and entry of new substrate/medium (see Fig. 3B). Regardless of fermenter and process type used, various process parameters have to be controlled during fermentation, such as substrate composition, cell densities, biomass shape, oxygen concentration, temperature, pH, shear forces, and foam formation (Rani & Rao 1999, Papagianni 2004). By the complexity of

the systems, strategies of mathematical techniques-experimental design and optimisation are used to achieve best operating conditions for growth of micro-organisms and product formation in order to obtain optimum yields (Kennedy & Krouse 1999, Rani & Rao 1999).

Solid state fermentation (SSF)

Solid state fermentation has a long tradition in Asian food industry, e.g. in production of the protein-rich Koji for sake brewing from rice. The long-standing experiences with SSF together with the huge human capital also influenced enzyme production in Asia – cellulases from fungal *Trichoderma* strains for example are won on large scale by the more work-intensive SSF (Hölker & Lenz 2005). In spite of the better established and standardised and therefore technically easier to handle SmF, a new trend towards fungal cultivation and enzyme production in SSF is seen also in Western countries (Raimbault 1998, Hölker et al. 2004, Rodríguez Couto & Sanromán 2005). Fungal morphology (cellular aggregation, mycelial pellet shape, size, etc.) very much influences production efficiencies in SmF and is often suboptimal in submerged culture of filamentous fungi that naturally prefer growth on surfaces (Papagianni 2004, Grimm et al. 2005, Kelly et al. 2006). SSF allowing the fungi to grow on surfaces therefore presents a more natural environment (Oojikaas et al. 2000, Aguilar et al. 2004). Higher oxygen availability is guaranteed by surface growth and by the fact that in SSF the space between micro-organisms and matrix is mostly filled with gas (Durand 2003). Carbon catabolite repression (repression by fermentable sugars) of enzyme synthesis is a problem in conventional SmF but not in SSF due to different nutrient and metabolite diffusion. Also due to different diffusion, stimulation by regulator molecules (inducers) is better in SSF and the product degradation is lower (Oojikaas et al. 2000, Aguilar et al. 2004, Viniestra-Gonzalez & Favela-Torres 2006). For all these and other reasons, productivity and enzyme yields in SSF can be higher than in SmF (Díaz-Godínez et al. 2001, Viniestra-González et al. 2003, Matsumoto et al. 2004, Sandhya et al. 2005, Shah & Madamwar 2005, Hongzhang et al. 2006, Patil & Dayanand 2006). Disadvantageous in SSF is, however, the lower thermal conductivity of the air space compared to the liquid enclosing micro-organisms in SmF. The better supply of oxygen in SSF therefore leads to a reduction in heat transfer and thus to problems in process up-scaling by difficulties in temperature control. In addition, in large scale application oxygen gradients will develop (Durand 2003, Hölker et al. 2004). SSF in general is hard to control, particularly in large plants. It is labour intensive and the contamination pressure is quite high (Cen & Xia 1999, Pandey et al. 2000). Different water absorption potentials of the distinct matrices used in SSF, mechanical stress exerted on the micro-organism in certain processes (see drum reactor in Fig. 2B), the need for a sterile or non-sterile process, general process control and up-scaling can put restraints on the reactor design (Mitchell et al. 2000, Durand 2003). In contrast, cultivation of mixed organisms is more easily

Table 1 Properties of bioreactor types used in SSF (Cen & Xia 1999, Durand 2003, Rodríguez Couto & Sanromán 2006b; compare also Fig. 2B)

Fermenter type	Oxygen transfer	Advantages	Disadvantages
Rotating drum reactor	By mixing substrate	Prevention of overheating Good oxygen/mass transfer	Clotting of substrate Low volume utilisation
Tray fermenter	By diffusion	Low investment Simple construction	Limited bed size (depths) by low oxygen transfer Up-scaling requires numerous trays and large chamber volumes
Column reactor, fixed-/packed-bed reactor	By air sparging the substrate	Relative simple construction Large bed size	Non-uniform growth Poor heat removal and difficult regulation of temperature and matrix water content
Fluidised-bed reactor	By pneumatic agitation of the substrate	Avoidance of adhesion and aggregation of substrate particles Equal temperature and humidity Continuous processes become possible	Damage to the inoculum through sheer forces

feasible in SSF which can be of advantage in production of enzyme mixtures and increasing absolute yields (Hölker et al. 2004; see below).

Also in SSF, the micro-organisms are cultured in different types of fermenters (Fig. 2B, Table 1), on a degradable (e.g. wheat bran) or non-degradable (e.g. polyurethane foam) matrix. The matrix serves the micro-organisms as anchor and, in the case of a solid substrate, also as nutrient source. Substrates for SSF can be solid by-products of agriculture and forestry such as rice grains, straw, wood chips and various other lignocellulosic wastes (Pandey et al. 2000, Rodríguez Couto & Sanromán 2006a). Costs estimated for the production of bulk enzymes by usage of such waste materials and the low budget technology for the regulation of SSF processes favor SSF over SmF. A further environmental advantage is that less waste water is generated (Aguilar et al. 2004, Hölker et al. 2004). Weighting its advantages and disadvantages (Table 1), views on whether SSF is adequate for bulk enzyme production are currently quite controversial but for special products, e.g. biopharmaceuticals, organic acids and certain enzymes, SSF might be more practical than SmF (Pandey et al. 2000). SSF is, however, not restricted to applications in productions. Instead of treating the raw materials with xylanase solutions for

biopulping and biobleaching (Daneault et al. 1994, Beg et al. 2001), fungal SSF can serve as an integral part in the process from compact lignocellulosic material to pulp and paper (Martínez-Íñigo et al. 2000, Gutiérrez et al. 2001, Helmy & El-Meligi 2002, Kang et al. 2003, Szendefy et al. 2003, van Beek et al. 2007). In order to replace petrochemicals and help to solve the problems of global warming, SSF with fungi on lignocellulosic substrates has also found high interest in biorefinery as an environmentally-friendly production system for fuels, energy, and chemicals from biomass (Tengerdy & Szakacs 2003, Watanabe 2007; see Chapters 6 and 22 of this book). Mushroom production on lignocellulosic wastes is also a form of SSF (see Chapter 22 of this book).

Cellulases

Hydrolytic enzymes cleaving β -1,4-glycosidic bonds in cellulose are known as cellulases. Three main types of cellulases are distinguished: i. endoglucanase (endo-1,4- β -glucanase, cellulase, EC 3.2.1.4), ii. exoglucanase (glucan 1,4- β -glucosidase, EC 3.2.1.74; cellulose 1,4- β -cellobiosidase, EC 3.2.1.91), and iii. β -glucosidase (cellobiase, EC 3.2.1.21). Endoglucanases cut the cellulose chain internally leaving oligosaccharides, which are cleaved by exoglucanases into cellobiose molecules (cellulose 1,4- β -cellobiohydrolase) or, glucose is chipped from their ends (glucan 1,4- β -glucosidase). β -glucosidases can react on cellobiose, thereby releasing glucose molecules (Esterbauer et al. 1991, Lynd et al. 2002; see Chapter 17 of this book).

Enzymatic systems of the various cellulolytic micro-organisms are however more diverse, acting variably on amorphous and crystalline cellulose – endoglucanase on the amorphous and exoglucanase on the micro-crystalline region of cellulose molecules (see Chapter 17 of this book). Synergistic effects between different types of cellulolytic enzymes were observed, topping in activities the sum of those of the individual enzymes (Lynd et al. 2002). In nature, cellulosic materials are processed by mixed cultures of cellulolytic organisms and in synergisms with many non-cellulolytic bacteria and fungi. The ability to decompose cellulose is widespread in bacteria in the orders Actinomycetales and Clostridiales. In fungi, it ranges from the phylum of Chytridiomycetes to the phylum of Basidiomycetes. Complex cellulase systems (known as cellulosome) bound to the cell via specific polypeptides exist in anaerobic bacteria (e.g. *Bacteroides*, *Clostridium*, *Ruminococcus*), non-complex systems of free extracellular enzymes in aerobic bacteria (e.g. *Cellulomonas*, *Thermobifida*) and also in some anaerobic bacteria (Lynd et al. 2002, Rabinovich et al. 2002, Zhang & Lynd 2004). Evidences for cellulosomes are found in some anaerobic rumen fungi such as *Neocallimastix*, *Pyromyces*, and *Orpinomyces* species (Shoham et al. 1999, Harhangi et al. 2003, Steenbakkers et al. 2003, Ximenes et al. 2005) whilst aerobic fungi express mixtures of free enzymes (Lynd et al. 2002, Rabinovich et al. 2002). Bacteria and fungi are both used in batch, fed-

Table 2 Examples of cellulase production by micro-organisms in batch processes (based on Tholudur et al. 1999, Sukumaran et al. 2005)

Organism	Production conditions (process, reactor, volume)	Substrate
Bacteria		
<i>Bacillus subtilis</i>	SSF, column reactor, 500 ml	Soybean industry residues
<i>Rhodothermus marinus</i>	SmF, stirred vessel, 150 l	Carboxymethyl cellulose
<i>Streptomyces</i> sp. T3-1	SmF, stirred vessel, 50 l	Carboxymethyl cellulose
Ascomycetes and related anamorphic genera		
<i>Melanocarpus albomyces</i>	SmF, stirred vessel, 700 l	Solka-Floc (powdered cellulose)
<i>Penicillium decumbans</i>	SSF, tray fermentor, 50 l	Wheat straw, bran
<i>Penicillium occitanis</i>	SmF, stirred vessel, 20 l	Paper pulp
<i>Thermoascus auranticus</i>	SSF, rotating drum, 10 l	Wheat straw
<i>Trichoderma reesei</i>	SmF, stirred vessel, 22 l	Steam pretreated willow
(= <i>Hypocrea jecorina</i>)	SmF, stirred vessel, 5 l	Corn steep liquor + lactose + xylose
	SSF, tray fermentor, 12,000 l	Corn cob residues
Basidiomycetes		
<i>Phanerochaete chrysosporium</i>	SmF, stirred vessel, 100 l	Cellulose

batch and continuous processes, either in SmF or SSF, for commercial production of cellulases (Bhat & Bhat 1997, Cen & Xia 1999, Tholudur et al. 1999, Sukumaran et al. 2005; Table 2) for applications in food and beverage industries (baking, malting, brewing, extraction processes), in animal feed and pharmaceutical industry, in the wood and textile industry (Nevalainen 1994, Watanabe et al. 2000, Galante & Formantici 2003; see Chapter 18 of this book), and potentially in ethanol production from lignocellulose (Sun & Cheng 2002, Gray 2007; see Chapter 6 of this book). According to Tolan & Foody (1999), the annual consumption of cellulases in the 1990s derived from submerged cultivation comprised an amount of 23,000 t valued \$US 125 million. At the time, it represented more than 10% of the total industrial enzyme market. Nevertheless, since production costs in SSF compared to SmF are low (\$US 0.2/kg versus \$US 20/kg at the time), crude cellulase production by SSF can have economical advantages (Tengerdy 1996).

Production of fungal cellulases

In submerged cultures in industrial production, mainly aerobic strains of the anamorphic genera *Trichoderma*, *Humicola*, *Aspergillus*, and *Penicillium* are used with volumes of up to a few hundred liters (Tolan & Foody 1999). Usually, mixtures of different enzymes are produced by the fungi (Martin et al. 2007) - an overview of different commercially available cellulases of *Trichoderma* and *Aspergillus* is given by

Nieves et al. (1998). In SmF, mostly complex media consisting of residual materials of different carbohydrates as C-source (examples are given in Table 2) and corn steep liquor, yeast extract or peptone as N-source are used in combination with vitamins, various salts, and other minerals (Tholudor et al. 2001, Tolan & Foddy 1999, Lynd et al. 2002, Sukumaran et al. 2005; Table 2). For efficient cellulase production, fungi typically need inducers, i.e. molecules that cause an activation of promoter sequences for transcription of the genes that are controlled by these promoters. Various types of inducers of cellulase production have been described: disaccharides (cellobiose, sophorose, gentiobiose, lactose), as well as cellulose itself and its oligosaccharides, which can be degraded into soluble sugars (Tolan & Foody 1999, Aro et al. 2005, Sukumaran et al. 2005). Of the simple sugars, in industrial production only lactose in milk whey as a by-product of the dairy industry is economically expedient as soluble inducer of cellulase expression. Other comparably cheap inducers are the purified powdered cellulose Solka-Floc obtained from delignification of wood and other fibre biomass, as well as spent liquors from paper mills and sugar industries containing several soluble and/or insoluble sugars (Tolan & Foody 1999, Lynd et al. 2002). To avoid hydrolysis of the natural inducers by the produced cellulases, resistant disaccharide analogs may be used where the oxygen in the *O*-glycosidic linkage is replaced by sulphur. However, such analogs are difficult to synthesise (Suto & Tomita 2001).

Cellulosic material used in SmF at higher concentrations has dual functions, serving i. as a C-source for fungal growth, and ii. as an inducer for enzyme production. Normally in fungi, there is low level constitutive expression of cellulases which leads to a release of oligosaccharides from available cellulosic material. Subsequently, these oligosaccharides act as inducers for higher levels of cellulase expression. Thereafter, the induced and secreted cellulases degrade any accessible cellulose to oligosaccharides and glucose, until larger amounts of liberated glucose cause catabolite repression of cellulase expression (Suto & Tomita 2001). Production in continuous cultivation has the advantage to reduce the catabolite repression caused by accumulation of reducing sugars (Sukumaran et al. 2005).

Because cellulases are bulk enzymes, cheap starting materials for microbial growth are preferred. In SSF, cheap solid cellulose-containing materials from agriculture and forestry are used as carbon source (Sukumaran et al. 2005). Cen & Xia (1999) list potential raw materials ranging from straw to various fibre and timber materials together with their carbohydrate, lignin, protein and ash contents. Due to the complexity, crystalline structure, and lignin content of such substrates, a problem in SSF is the slow colonisation of the raw materials by the micro-organisms. Only some feedstock (bran from various cereals, corn cobs, wastes from the pulp and paper industry) can be used directly. More complex materials need conditioning by diluted acids, lime, pretreatments by steam explosion or hydrothermal processes in order to give easier accessible substrates (Lynd et al. 2002).

Applications of fungal cellulases are various (see above and Chapter 17 of this book) and may require specific cellulases or mixtures of enzymes of different purities and properties. The choice of substrate(s) as well as the fungal organisms influences the mixtures of cellulases that will be produced (Lynd et al. 2002). Combinations of strains of different species (e.g. *Trichoderma* and *Aspergillus* strains) may in some instances be preferable (Vyas & Vyas 2005, Wen et al. 2005). By recombinant DNA technology, it is also possible to produce individual enzymes of choice in heterologous hosts, for example in the bakers yeast *Saccharomyces cerevisiae*. Cellulase genes cloned for such purposes in *S. cerevisiae* have their origin in bacteria, fungi, and plants (Lynd et al. 2002, Saloheimo 2004). Through introduction of foreign cellulose genes, it is even possible to make the yeast growing on pure cellulose (den Haan et al. 2007).

Xylanases

One of the main hemicelluloses in plants is xylan, a linear polymer of β -1,4-linked xylose, that functions in secondary cell walls as a bond between lignin and cellulose (Tímell 1967, Wong et al. 1988). Depending on its origin, different substituents can be attached to the xylan backbone [for a more detailed illustration, see de Vries & Visser (2001), Beg et al. (2001) and Chapter 17 of this book]. Two main types of xylanases can be distinguished (Polizeli et al. 2005): endo-1,4- β -xylanase (EC 3.2.1.8) and xylan 1,4- β -xylosidase (EC 3.2.1.37), both cleaving the β -1,4-glycosidic bonds in xylan. Endo-1,4- β -xylanase thereby decreases the polymerisation level of the xylan backbone whereas xylan 1,4- β -xylosidase acts on smaller xylooligosaccharides and xylobiose to give β -D-xylopyranosyl residues. Other xylanolytic enzymes are acetylerase (EC 3.1.1.6), different types of arabinase (α -N-arabinofuranosidase, EC 3.2.1.55; arabinan endo-1,5- α -L-arabinosidase, EC 3.2.1.99), xylan α -1,2-glucuronidase (EC 3.2.1.131) and feruloyl esterase (EC 3.1.1.73) (Beg et al. 2001, Polizeli et al. 2005; see also Chapter 17 of this book). Xylanases have a range of industrial applications (removing hemicellulosic residues in animal feed, improving dough quality in bakery, improving juice extraction from fruits and vegetables in the food industry) but are mainly used for bleaching in the pulp and paper industry (Polizeli et al. 2005; see Chapter 17 of this book). They are assumed to cleave bonds between lignin and cellulose (Paice et al. 1992, reviewed by Uffen 1997, Polizeli et al. 2005) that might help to protect against cellulolytic degradation and hinders the exposure of lignin to bleaching compounds (Viikari et al. 1994, Beg et al. 2001; see Fig. 4 in Chapter 21 of this book).

Xylanases are predominantly produced by micro-organisms (Table 3), but are also found in marine algae, protozoans, crustaceans, insects, snails, and seeds of land plants (Sunna & Antranikian 1997). As in the case of cellulases, microbial production of xylanases can take place in SmF and SSF (see Table 3), although about 80-90% of the commercial xylanases are gained through submerged ferment-

Table 3 Examples of xylanase production by micro-organisms in batch processes

Organism	Production conditions (process, substrate)	Maximum yield	Reference
Bacteria			
<i>Bacillus circulans</i>	SmF, sugarcane bagasse hydrolysate	8.4 U/ml	Bocchini et al. 2005
<i>Bacillus</i> sp.	SSF, wheat bran	720 U/g dry substrate	Gessesse & Mamo 1999
<i>Streptomyces</i> sp.	SSF, wheat bran	2360 U/g dry substrate	Beg et al. 2000
	SSF, <i>Eucalyptus</i> kraft pulp	1200 U/g dry substrate	
Ascomycetes and related anamorphic genera			
<i>Aspergillus amowari</i>	SmF, wheat bran	28.3 U/ml	Li et al. 2006
<i>Aspergillus foetidus</i>	SmF, oat spelt, xylan	322 U/ml	Chipeta et al. 2005
<i>Aspergillus niger</i>	SSF, rice straw	5070 U/g dry substrate	Kang et al. 2004
<i>Aspergillus phoenicis</i>	SmF, spent sulphite liquor	173 U/ml	Chipeta et al. 2005
<i>Melanocarpus albomyces</i>	SmF, wheat straw	172 U/ml	Saraswat & Bisaria 1997
	SSF, wheat straw	7760 U/g dry substrate	Narang et al. 2001
<i>Paecilomyces thermophila</i>	SSF, wheat straw	18,500 U/g dry substrate	Yang et al. 2006
<i>Penicillium oxalicum</i>	SmF, wheat bran	16.1 U/ml	Li et al. 2007
<i>Trichoderma harzianum</i>	SmF, birchwood xylan	44.9 U/mg protein	Seyis & Aksoz 2005
(= <i>Hyprocrea lixii</i>)	SSF, sugarcane bagasse	288 U/ml	Rezende et al. 2002
<i>Trichoderma longibrachiatum</i>	SmF, Solka-Floc	272 U/ml	Royer & Nakas 1989
	SSF, wheat bran	5.01 U/g dry substrate	Kovacs et al. 2004
<i>Trichoderma reesei</i>	SmF, beech xylan	9 U/ml	Bailey et al. 1993
(= <i>Hypocrea jeconina</i>)	SSF, rice straw	122 U/ml	Colina et al. 2004
Basidiomycetes			
<i>Schizophyllum commune</i>	SMF, microcrystalline cellulose (Avicell)	4839 U/ml	Haltrich et al. 1993
	SSF/SmF, spruce sawdust	0.37 U/ml	Paice et al. 1978
Mixed cultures			
<i>A. phoenicis</i> and <i>T. reesei</i>	SSF, bagasse	714 U/g dry substrate	Duenas et al. 1995
<i>A. niger</i> and <i>T. reesei</i>	SSF, soymeal	2800 U/g dry substrate	Gutierrez-Correa & Tengerdy 1998

tation, usually with filamentous ascomycetes and their related anamorphic genera (Polizeli et al. 2005, Chávez et al. 2006; Table 3). Species are often the same than those used in cellulase production (compare examples in Tables 2 and 3). Fungi tend to produce mixtures of xylan-degrading enzymes, different xylanases combined with accessory xylanolytic enzymes for debranching of substituted xyans (Haltrich et al. 1996, Chávez et al. 2006) that without further downstream processing might directly be applied in biobleaching of paper pulp (Szendefy et al. 2006). Important for this is that the enzymes are as much as possible cellulase-free (Christov et al. 1999). Also for applications in the textile industry, presence of cellulases is unwanted in xylanase preparations in order not to damage the cellulose (Kulkarni et al. 1999, Polizeli et al. 2005). Production conditions, in particular media components, can favour which types of enzymes are obtained from the fungi and help to avoid or reduce impurities with unwanted enzymes (Haltrich et al. 1996).

Production of fungal xylanases

Bulk substrates used for SmF and SSF are comparable to the ones used for cellulase production (Tables 2 and 3), except that in most instances cellulose is replaced by xylan and its sugar xylose in order to induce production of the required xylanases and to avoid unwanted induction of cellulase production. Various derivatives of xylan (xylose, xylobiose, xylooligosaccharides, etc.) are possible inducers of xylanases although effects on xylanase biosynthesis vary with the species used for production (Kulkarni et al. 1999). In natural production of xylanase, isolated xylan from different lignocellulosic materials (beechwood, birchwood, larchwood, oat spelt) and other agricultural and forestry residues (e.g. beet pulp, corn cobs, wheat/rice bran, straw) are often used as inducing substrate. In SmF, the concentration of the xylan substrate can be as high as 75 g/l (Haltrich et al. 1996). Measures to optimise natural xylanase production and scale up the processes are principally the same as in cellulase production. Natural production of xylanases can be improved by altering culturing conditions such as concentrations of media components (carbon, nitrogen, salts, etc.), temperature, pH, aeration, and others. Also for establishing large scale xylanase productions, modern experimental design with statistical methodologies are used to calculate best process parameters (Katapodis et al. 2006, Li et al. 2006, 2007, Azin et al. 2007). In addition, mutants with better xylanase yields are screened for (Smith & Wood 1991, Park et al. 2002, Hao et al. 2006).

In cases where pure enzymes are required and where by culture conditions it is not possible to produce pure xylanases with a filamentous fungus without cellulase contamination, fungal xylanase genes are expressed heterologously in *Escherichia coli* and, more often, in yeasts such as *Pichia pastoris* and *S. cerevisiae* (Hahn-Hägerdal et al. 2005, Jeffries 2006, Korona et al. 2006, Berrin et al. 2007, Chen et al. 2007, Tung et al. 2007). Expression in organisms well established for industrial heterologous protein production has the further advantage that fermentation conditions

are easily adapted to xylanase production (Damaso et al. 2006). However, it is also possible to enhance enzyme yields in homologous and heterologous filamentous fungi by replacing the natural xylan-inducible promoter by more efficiently working promoters underlying different regulation schemes (de Faria et al. 2002, Rose & van Zyl 2002, Levasseur et al. 2005). Recombinant protein production has another positive consequence for applications: site-directed mutagenesis and directed evolution are used in molecular gene engineering to optimise the enzyme properties (see for examples on xylanases see Xion et al. 2004, Fenel et al. 2006, Sriprang et al. 2006, Stephens et al. 2007).

Laccases

Laccases (EC 1.10.3.2) are oxidoreductases capable of oxidising phenols as well as aromatic amines (Leonowicz et al. 2001; see Chapter 17 of this book). Because of their wide substrate range, these enzymes are attractive for various biotechnological applications, for example in the food industry, the textile industry, the pulp and paper industry, in wood composite production, in soil bioremediation, and others (Hüttermann et al. 2001, Mai et al. 2004, Husain 2006, Kilaru 2006, Rodríguez Couto & Toca Herrera 2006; see also Chapters 17 and 18 of this book).

Laccases have four copper atoms as cofactors located at the catalytic centre of the protein and they belong therefore to the group of multicopper oxidases. In nature, these enzymes are produced by many fungi and plants, but also by a few bacteria and insects (Mayer & Staples 2002, Claus 2003, 2004, Baldrian 2006, Hoegger et al. 2006). Besides laccase from the lacquer tree *Rhus vernificera* (Johnson et al. 2003), commercially available laccases are usually of fungal origin (see Table 4), most likely from white-rot and saprophytic basidiomycetes such as *Trametes*, *Pleurotus* and *Agaricus* species that secrete high amounts of these enzymes (Minussi

Table 4 Examples of natural production of laccases by fungi

Organism	Production conditions* (process, reactor, volume)	Yield	Reference
Ascomycetes			
<i>Botryosphaeria</i> sp.	SmF, flask, 125 ml	5.4 U/ml	Vasconcelos 2000
<i>Botrytis cinerea</i>	SmF, stirred vessel, 10 l	28 U/ml	Fortina et al. 1996
<i>Coniothyrium minitans</i>	SmF, stirred vessel, 20 l	55.2 U/ml**	Dahiya et al. 1998
<i>Monotropa</i> sp.	SmF, flask, 250 ml	13.55 U/ml	Wang, J.W. et al. 2006

* Batch cultivation unless otherwise noted

** For better comparison, enzyme activities given in the original literature in nkat units were transformed into international units (IU) through division by a factor of 16.67, and activities given in arbitrary units (AU) by a factor of 36, respectively (see Kilaru 2006 for further explanations)

Table 4 continued

Organism	Production conditions* (process, reactor, volume)	Yield	Reference
Basidiomycetes			
<i>Fomes</i>	SmF, flask, 2 l	20.29 U/ml	Papinutti & Martínez 2006
<i>sclerodermeus</i>			
<i>Funalia trogii</i>	SmF, flask, 250 ml	4.9 U/ml	Kahraman & Gurdal 2002
<i>Grifola frondosa</i>	SSF, bottles, 850 ml	6 U/ml	Xing et al. 2006
<i>Lentinus tigrinus</i>	SSF, flask, ?	30 U/g dry substrate	Lechner & Papinutti 2006
<i>Nematoloma frowardii</i>	SSF, flask, ?	0.5 U/ml	Hofrichter et al. 1999
<i>Panus tigrinus</i>	SmF, flask, 200 ml	0.05 U/ml**	Chernykh et al. 2005
	SmF, bubble column, 3 l	4.3 U/ml	Fenice et al. 2003
	SmF, stirred vessel, 3 l	4.6 U/ml	
	SSF, rotating drum, 20 l	1.3 U/ml	
<i>Phlebia floridensis</i>	SmF, flask, 100 ml	10.4 U/ml	Arora & Gill 2005
<i>Pleurotus ostreatus</i>	SmF, flask, 250 ml	22.3 U/mg protein**	Prasad et al. 2005b
<i>Pleurotus pulmonarius</i>	SSF, flask, 250 ml	24.4 U/g	de Souza et al. 2006
<i>Pycnoporus cinnabarinus</i>	SmF, flask, 250 ml	266.6 U/ml	Lomascolo 2003
<i>Pycnoporus sanguineus</i>	SmF, flask, 250 ml	29 U/ml	Herpoël et al. 2000
<i>Trametes gallica</i>	SSF, flask, ?	46.5 U/g dry substrate	Vikineswary 2006
<i>Trametes hirsuta</i>	SmF, flask, 250 ml	8.6 U/ml	Dong et al. 2005
	SSF, immersion reactor***, 500 ml	2.2 U/ml	Rodríguez Couto et al. 2004b
	SSF, flask, 250 ml	5.4 U/ml**	Rosales et al. 2005
	SmF, stirred vessel, 10 l	80.7 U/ml	Koroleva et al. 2002
	batch + fed-batch	+ 83.8 U/ml	
<i>Trametes modesta</i>	SmF, flask, 300 ml	10.7 U/ml**	Nyanhongo et al. 2002
<i>Trametes pubescens</i>	SmF, stirred vessel, 20 l	330 U/ml	Galhaup et al. 2002
	batch + fed-batch	+ 743 U/ml	
<i>Trametes</i> sp.	SmF, flask, 300 ml	20.0 U/ml	Jang et al. 2002
<i>Trametes trogii</i>	SmF, flask, 250 ml	22.75 U/ml	Trupkin et al. 2003
<i>Trametes versicolor</i>	SmF, stirred vessel, 100 l	70 U/hl	Fähræus & Reinhammar 1967
	SmF, flask, 100 ml	5.2 U/ml	Mikiashvili et al. 2005
	SmF, bubble column, 2 l	4 U/ml	Domínguez et al. 2007
	SSF, tray, 17 l	3.5 U/ml	Rodríguez Couto et al. 2003
<i>Ganoderma</i> sp.	SmF, flask, 250 ml	692 U/ml	Revankar & Lele 2006

*** Immersion reactor: in such systems, micro-organisms are grown on an inert matrix or a solid substrate which are periodically immersed into culture medium (Rodríguez Couto et al. 2002b)

et al. 2002, Xu 2005). Usually, commercial laccases come from SmF fermentation (Cherry & Fidantsef 2003; Table 4).

Next to various basidiomycetes, many ascomycetes are also producers of interesting laccases (Baldrian 2006, Kilaru 2006; Table 4). Although laccases usually have a wide substrate spectrum, enzymes can differ in the range of substances they attack (Kilaru 2006, Mander et al. 2006). In consequence, enzymes may be differentially suitable for specific tasks in biotechnology. Most enzymes are (best) active in the lower pH range (pH 2–4; Baldrian 2006, Kilaru 2006) but applications may ask for reaction optima at higher pHs in the neutral or slightly alkaline range. Other catalytic properties (e.g. a high redox potential, temperature optimum) and protein stabilities play also a role in applications. In the literature, over 100 laccases have been described in more or less detail at the enzymatic level. Nevertheless, screening for new enzymes is still intensively going on in order to find enzymes better adapted to the respective biotechnological problems (Baldrian 2006, Kilaru 2006).

Production of native laccases in pure culture

The most studied genus concerning laccase production is probably *Trametes* covering numerous of the strong white-rot fungi. Several *Trametes* species have been tested for their ability to produce laccases, either in submerged or in solid state production. Although production by SmF is best established, results show that SSF also has its potential in efficient laccase production (Tables 4 and 5). As with the other enzymes, an important factor in production is the cultivation medium. Both, the sources of carbon and nitrogen as well as relative amounts of the nutrients play a role. In *T. versicolor*, cellobiose and mannitol gave better results than glucose (Mikiashvili et al. 2005) and carbon limitation (glucose depletion) acts stimulating in laccase production (Tavares et al. 2005). However, for best results

Table 5 Laccase production by *Trametes* species grown in SSF

Organism	Reactor type	Substrate/ matrix	Yield [U/ml]	Reference
<i>Trametes hirsuta</i>	Tray	Grape seeds	18.0*	Rodríguez Couto et al. 2006
		Nylon sponge	6.0*	
	Immersion reactor	Grape seeds	12.0*	Rodríguez Couto et al. 2002a
<i>Trametes versicolor</i>	Immersion reactor	Barley bran with	0.6	
	Column reactor	glucose medium	0.6	
	Tray		3.5	
	Bubble column	Glucose medium	1.7	Rancaño et al. 2003

*For better comparison, nkat units were transformed into international units (IU) through division by a factor of 16.67 (see Kilaru 2006)

sugar concentrations have to be adjusted to reach a balance between fungal biomass and enzyme production (Ryan et al. 2007). In continuous cultivation, this can be achieved by constant feeding of low glucose amounts (Galhaup et al. 2002).

Plant waste material (e.g. mandarine peels, groundnut shells) and organic nitrogen sources (corn steep liquor, peptone, casein hydrolysate) perform well in cultivation with *Trametes* and also *Pleurotus* species (Dong et al. 2005, Mikiashvili et al. 2005, 2006). Nitrogen supplementation in SSF of selected agro- and forestry-wastes (saw dust, oil palm frond, wheat straw, beech tree leaves) can very much raise enzyme yields of various white-rot fungi (D'Souza et al. 1999, Kachlishvili et al. 2006, Vikineswary et al. 2006). Other studies report N-limitation to be better for laccase production in *P. ostreatus* (Hou et al. 2004) and *Pycnoporus cinnabarinus* (Eggert et al. 1996). On a superficial view, results may appear to be contradictory (Galhaup et al. 2002). Effects of nitrogen addition depend however on both strain and substrate (Kachlishvili et al. 2006). Other factors also play a role. Static cultures of *P. ostreatus* and *Trametes gallica* were reported to be superior to agitated cultures (Hou et al. 2004, Dong et al. 2005), whilst in *Irpex lacteus* no differences were seen (Kasinath et al. 2003, Shin 2004), and in *Funilia trogii* an agitated culture outperformed a static one (Birhanli & Yesilada 2006). Also culture volumes have an influence. Transfer of established conditions from laboratory to larger scales of submerged culture therefore remains a problem. Fungal morphology is likely to be different in bioreactors of increased size or different shape due to altered aeration conditions (being of special importance in fermentations where production and application of the oxygen-dependent enzymes occur simultaneously such as in waste water treatment) and due to the shearing forces applied in the different reactor types (Hess et al. 2002, Bermek et al. 2004, Ryan et al. 2005). Immobilisation of fungi on solid matrices is a very active research area to overcome problems with up-scaling in production of laccases by the various white-rot fungi. Several solid matrices have successfully been tested as support for laccase production by SSF – partially in combined procedures targeting at degradation of toxic and recalcitrant compounds in liquids: stainless steel sponge, nylon sponge, polyurethane foam and alginate beads (Rodríguez Couto et al. 1997, 2004a,b, Prasad et al. 2005a, Domínguez et al. 2007; see Table 5 for examples) and several agricultural residues, like kiwi fruit wastes (Rosales et al. 2005), barley bran (Rodríguez Couto et al. 2002a), grape seeds and stalks (Lorenzo et al. 2002, Moldes et al. 2004), and corn cob (Tychanowicz et al. 2006). Optimisation in growth and laccase production in SmF cultures is nowadays addressed by experimental design technologies considering variable parameters like nutrient concentration, inducers, agitation, pH and inoculum (Prasad et al. 2005b, Tavares et al. 2006). The pH has been shown to influence laccase yields e.g. in *Botrytis cinerea* - at pH 3.5, 20% more laccase was produced than at pH 5.0 (Fortina et al. 1996). In addition, the temperature of cultivation can have an influence (Forina et al. 1996, Koroleva et al. 2002,

Nyanhongo et al. 2002, Wang, J.W. et al. 2006). For example, in glucose-based medium, an increase from 28°C to 37°C resulted in a decrease of laccase yield in cultivation with *Trametes* sp., whereas in cellobiose medium the laccase activity in the supernatant increased more than two times with such temperature shift (Tong et al. 2007).

As with other enzymes acting in lignocellulose degradation, small amounts of extracellular laccases are often constitutively secreted (Bollag & Leonowicz 1984, Koroljova-Skorogbogot'ko et al. 1998, Scheel et al. 2000, Da Cunha et al. 2003, Zhang, M. et al. 2006). Higher yields however require induction of expression. Several inducers have been applied to increase yields of laccase: copper, phenols, 2,5-xylidine and related compounds in *T. versicolor* (Collins & Dobson 1997, Tavares et al. 2005, Kollmann et al. 2005, Domínguez et al. 2007, Ryan et al. 2007), ethanol in *P. annabarinus* (Lomascolo et al. 2003, Meza et al. 2006, 2007), aromatic compounds including veratryl alcohol in *Botryosphoraeria* sp. (Vasconcelos et al. 2000, Dekker & Barbosa 2001), copper and phenolic compounds in *Panus tigrinus* (Chernykh et al. 2005), copper, cotton stalk extract, dimethyl sulphoxide, and the synthetic substrate ABTS [2,2'-azino-di-(3-ethylbenzothiazolin-6-sulphonic acid)] in *P. ostreatus* (Ardon et al. 1996, Palmieri et al. 2000, Hou et al. 2004, Shah et al. 2006), and copper and lignin-related compounds in *Pleurotus pulmonarius* (Souza et al. 2004, Tychanowicz et al. 2006). Complex substrates such as sugar cane bagasse and lignocellulosic material such as wood and peanut shells have also been shown to be effective in various fungi (D'Souza et al. 1999, Arora & Gill 2001, Linke et al. 2005, Makela et al. 2006). Many fungal strains produce a range of laccase isoenzymes, being either variations of differentially glycosylated laccases from the same gene or enzymes from different genes (for examples see Palmieri et al. 1997, 2000, 2003, Dong et al. 2005, D'Souza-Ticlo et al. 2006, Linke et al. 2005, Lorenzo et al. 2006; see Chapter 17 of this book). Induction can be selective on the different laccase genes as shown for example for *Trametes* sp. I-62 and *Pleurotus sajor-caju* (Soden & Dobson 2001, Terrón et al. 2004).

Inducers of laccase production might be toxic (copper, 2,5-xylidine) and/or expensive (ABTS). Laccase has been produced with *Trametes villosa* and *T. versicolor* strains in the 500 l stirred vessel shown in Fig. 3A using 0.6 mM 2,5-xylidine as an inducer in an artificial medium I (per l: 10 g glucose, 0.5 g yeast extract, 2.5 g L-asparagine, 0.15 g DL-phenylalanine, salts; Shekholeslani 1991) developed for high laccase production. However, the toxic 2,5-xylidine can be replaced by kraft lignin (Indulin AT, Westvaco, Raleigh, USA). Yields of laccase obtained with medium I with the *Trametes* strains are in the range of 12 U/ml (Shekholeslami 1991; M. Euring & A. Kharazipour, unpublished). The laccase has been used in the production of MDF (medium density fibreboards) and particle boards in laboratory and technical scale at concentrations of 20 U/g raw material (dry process) and 544 U/g (wet process) (Kharazipour 1996, Kharazipour et al. 1997; further reading in Chapter 18 of this book).

Laccase production in mixed cultures

Interactions between different fungi as well as between fungi and certain prokaryotes can have an inducing effect on laccase activity, often in strain-specific manners (Iakovlev & Stenlid 2000, Baldrian 2004, Ferreira Gregorio et al. 2006, Chi et al. 2007, Kleeman 2007; Fig. 4). Such effects of co-cultivation might help individual species and microbial communities in lignin degradation but also in competition and defence of their habitats (Boddy 2000, Iakovlev & Stenlid 2000). Several white-rot and litter-degrading basidiomycetes showed higher laccase activity in cultivation with another basidiomycete species on plates and in liquid medium (Iakovlev & Stenlid 2000, Baldrian 2004, Baldrian & Šnajdr 2006, Ferreira Gregorio et al. 2006). Increases in laccase production up to 25fold have been described (Baldrian 2004). Likewise, *Trichoderma* species can stimulate laccase production of white-rot basidiomycetes in submerged and solid state cultures (Freitag & Morrell 1992, Savoie & Mata 1999, Hatvani et al. 2002, Velázquez-Cedeño et al. 2004, Zhang, H. et al. 2006). Stimulating laccase production between white-rot fungi might be useful in biopulping of wood (Wang, H.L. et al. 2006, Chi et al. 2007) and wastewater treatment (García-Mena et al. 2005). Laccase produced to larger

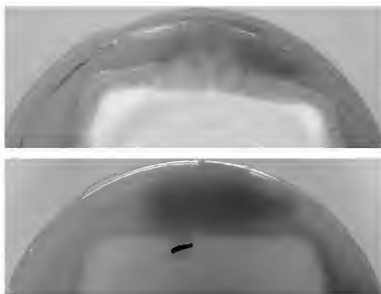


Fig. 4 Co-cultivation of *Coprinopsis cinerea* strain AmutBmut with an unknown bacterium on agar in a Petri-dish (shown from above to demonstrate the growth of the microorganisms and from below to document laccase activity by agar staining). The bacterium was streaked around the fungal mycelium (black mark = growth front at the time of bacterial inoculation) that in consequence was retarded in growth. Where the fungus managed to overcome the bacterial barrier, laccase has been produced. Laccase activity is seen by the staining of the agar through oxidation of the colourless substance ABTS converting it into a green-brown-coloured compound

amounts by mixtures of aquatic hyphomycetes can be a possible alternative in wastewater treatment (Junghanns et al. 2005). Whether co-cultivation of two species in SmF or SSF in bioreactors for the pure target of enzyme production and harvest is feasible, remains to be tested. General process management can be expected to be more complex under such circumstances.

Fungal laccase genes and their recombinant expression

Natural fungal strains can have several different laccase genes. Five non-allelic genes are currently known for the white-rots *T. villosa* and *P. sajor-caju*, four non-allelic genes in *P. ostreatus* and *T. versicolor*, three in *Lentinula edodes*, *P. cinnabarinus* and *Trametes* sp. I-62, and, in addition, three in the plant pathogen *Rhizoctonia solani* (Necochea et al. 2005, Hoegger et al. 2006). For very few of these, protein properties have been described (Table 6) - a reason for this might be the difficulty to separate the different isoenzymes coming from these genes, another that not enough enzyme is naturally produced from the genes by their hosts. Further to this, the saprophytic dung fungus *Coprinopsis cinerea* (formerly *Coprinus cinereus*) has in total seventeen different laccase genes (Kilaru et al. 2006a). In a standard nutrient-rich growth medium based on yeast- and malt-extract (Granado et al. 1997), no or only neglectable enzyme activity was encountered in *C. cinerea* cultures and only little, when copper was added (Fig. 5). 2,5-xyldine and veratryl alcohol were not effective as inducers of laccase production in this fungus (M. Navarro-González).

Table 6 Properties of laccases from known genes of white rot fungi (after Hoegger et al. 2006 and Kilaru 2006)

Organism	Laccase gene	pI	Optimal pH*				Opt. temperature [°C]
			ABTS	SGZ	DMP	Guaiacol	
<i>Ceriporiopsis subvermispora</i>	<i>lcs1</i>	3.6	-	-	-	-	-
<i>Pleurotus</i>	<i>pox2</i>	3.3	3.0	6.0	3.5	-	50-60
<i>ostreatus</i>	<i>poxa3</i>	4.1	3.6	6.2	5.5	-	35
<i>Pycnoporus</i>	<i>lac1</i>	<3.5	-	-	-	-	-
<i>cinnabarinus</i>	<i>lcc3-1</i>	3.7	-	-	-	4.0	-
<i>Trametes pubescens</i>	<i>lap2</i>	2.6	3.0	4.5	3.0	-	50-60
<i>Trametes villosa</i>	<i>lcc1</i>	3.5	≤ 2.7	5.0-5.5	-	-	-
	<i>lcc2</i>	6.2-6.8	6.0	5.0-5.5	-	-	-
<i>Trametes</i> sp. AH28-2	<i>lacA</i>	4.2	-	-	-	4.5	50
<i>Trametes</i> sp. 30	<i>lac1</i>	3.6	-	4.5-5.0	-	-	55

* substrates: ABTS = 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid), DMP = 2,6-dimethoxyphenol, SGZ = syringaldazine; -: not known

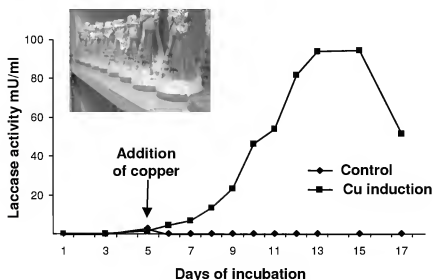


Fig. 5 Laccase production in 100 ml standing cultures of *Coprinopsis cinerea* strain AmutBmut at 37°C in yeast- and malt-extract medium with and without supplementation of 0.1 mM CuSO₄. Laccase activity was measured as described (Kilaru et al. 2006b)

lez, unpublished), although laccase production on protein-rich soybean-based medium has been reported (Yaver et al. 1999). Yields however were not high enough to purify and characterise the naturally produced enzyme Lcc1 beyond determining its molecular size (Schneider et al. 1999).

In order to obtain higher yields of individual laccases from basidiomycetes, several attempts have been made in the past to express selected laccase genes in ascomycetous yeasts (*Kluyveromyces lactis*, *Pichia methanolica*, *P. pastoris*, *S. cerevisiae*, *Yarrowia lipolytica*) and in filamentous ascomycetes and anamorphs (*Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae*, *T. reesei*), species that are well established in industrial fermentation (Table 7; for a complete literature overview see Kilaru 2006). However, these attempts were usually little successful since either no or only low enzymatic activity was obtained, stabilities of enzymes were low and sometimes also enzymatic properties altered (Berka et al. 1997, Sigoillot et al. 2004, Bulter et al. 2003, Piscitelli et al. 2005). First of all, the choice of the gene had an influence on the outcome of the experiments (Piscitelli et al. 2005, Kilaru 2006, S. Kilaru et al. unpublished). Secondly, expressed proteins often had altered glycosylation patterns (Otterbein et al. 2000, Madzak et al. 2005, Piscitelli et al. 2005), a post-translational protein modification that influences the protein properties. Glycosylation modes differ between different classes of eukaryotes (Brooks 2006). Obviously, there are also fundamental differences between asco- and basidiomycetes. Newer efforts in recombinant laccase production therefore concentrate on the basidiomycetes themselves (Alves et al. 2004, Kajita et al. 2004, Kilaru et al. 2006b). In *P. cinnabarinus*, yields as high as 280 U/ml were obtained from re-

combinant enzyme production under specific culture conditions, which is nearly as high as the best natural production rate by a basidiomycete (Alves et al. 2004; compare Tables 4 and 7). In *C. cinerea*, enzyme yields of 3 U/ml in recombinant production of *Lcc1* have been published (Kilaru et al. 2006b) – altering culturing conditions improved this in the meantime by a factor of 4 (Kilaru 2006, M. Rühl, unpublished; Table 7) into satisfactory levels (compare also Table 4). Currently it is unclear whether *P. cinnabarinus* is more efficient in overall laccase production than *C. cinerea*. Homologous genes were used in the expression studies with *P. cinnabarinus* (*lac1*) and *C. cinerea* (*lcc1*) and the different proteins could account for differences in yields (see below). The promoters used are possibly of higher influence – in *P. cinnabarinus* with two promoters from *Schizophyllum commune*, addition of ethanol increased laccase production by factors of more than 30. Without induction, yields of only 8–10 U/ml were achieved (Alves et al. 2004). A most recent study showed dual effects of ethanol on laccase production in *P. cinnabarinus*: increase of laccase gene transcription and inhibition of protease activities (Meza et al. 2007). Addition of ethanol to cultures of *C. cinerea* transformants unfortunately had no effect on laccase yields (Kilaru et al. 2006b).

Table 7 Recombinant expression of laccases in fungi

Laccase gene	Expression host	Yield	Reference
<i>lcs-1</i> of <i>Ceriporiopsis subvermispora</i>	<i>Aspergillus nidulans</i>	0.23 U/ml	Larrondo et al. 2003
<i>lcc1</i> of <i>Coprinopsis cinerea</i>	<i>Aspergillus oryzae</i> <i>C. cinerea</i>	135 mg/l 3–12 U/ml	Yaver et al. 1999 Kilaru 2006, Kilaru et al. 2006b, M. Rühl unpublished
<i>lcc5</i> of <i>C. cinerea</i>	<i>C. cinerea</i>	10–30 U/ml	Kilaru 2006, M. Rühl unpublished
<i>lac1</i> of <i>Melanocarpus albomyces</i>	<i>Trichoderma reesei</i>	15 U/ml*	Kiiskinen et al. 2004
MtL of <i>Myceliophthora thermophila</i>	<i>A. oryzae</i>	-	Xu et al. 1998
<i>lac1</i> of <i>Pycnoporus cinnabarinus</i>	<i>Pycnoporus cinnabarinus</i>	280 U/ml*	Alves et al. 2004
RSL of <i>Rhizoctonia solani</i>	<i>A. oryzae</i>	-	Xu et al. 1998
<i>lcc1</i> of <i>Trametes versicolor</i>	<i>Pichia methanolica</i> <i>Pichia pastoris</i>	12.6 U/ml 140 U/ml	Guo et al. 2005 Hong et al. 2002
<i>laccase III</i> of <i>T. versicolor</i>	<i>T. versicolor</i>	-	Kajita et al. 2004
<i>lacA</i> of <i>Trametes</i> sp.	<i>P. pastoris</i>	8.3 U/ml	Hong et al. 2006

* nkat units were divided by a factor of 16.67 into IU (see Kilaru 2006)

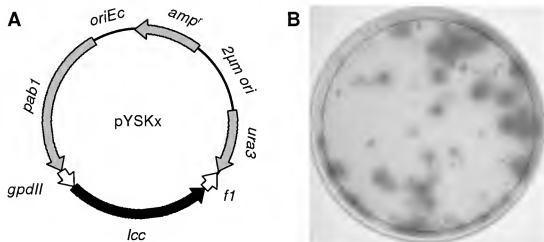


Fig. 6. Transformation of laccase genes in *Coprinopsis cinerea*. A. Transformation vector of the series pYSKx with a basidiomycete laccase gene *lcc* under control of the heterologous *Agaricus bisporus gpdII* promoter. *pab1* encodes para-aminobenzoic acid synthase and acts as a marker gene for selection in *C. cinerea pab1* auxotrophs. *oriEc* is the start point (origin) of DNA replication in *Escherichia coli*, *amp^r* a selection marker gene that mediates ampicillin resistance in *E. coli*, and *f1* an *E. coli* phage replication origin useful for single-strand DNA synthesis. *2μm ori* is a replication origin for the yeast *Saccharomyces cerevisiae* and *ura3* a selection marker gene for orotidine 5'-phosphate decarboxylase that complements certain uracil auxotrophs of yeasts. Bacterial and yeast sequences in this vector are required for gene cloning purposes (further details in Kilaru et al. 2006b,c). B. Once a vector with a subcloned laccase gene (here pYSK7 with gene *lcc1* behind *gpdII* promoter) has been transformed into *C. cinerea*, positive transformants secrete functional laccase into the medium about 4 to 5 days later. ABTS added to the medium will be converted into a greenish stain, marking the positive clones by large halos that form around the colonies (larger dark spots in the photo). Note in the photo, transformed clones are just starting to grow. Transformants failing to produce laccase are seen only as small spots of pin-head size without halo

In contrast to *P. cinnabarinus* (Alves et al. 2004), *C. cinerea* is a readily transformable basidiomycete (Binnering et al. 1987, Granado et al. 1997) and therefore represents by this property an excellent choice as a host for overexpression of individual laccases, both from own genes as well as from genes of other species (Kilaru et al. 2006b,c, Grimrath 2007). Screening for suitable transformants is fast (Fig. 6) and the large numbers of transformants obtained from a single transformation experiment eases to find the best producers (Kilaru et al. 2006b). In the future, this should allow to easily analyse tailored enzymes with optimised properties produced either by targeted or by random mutagenesis. Currently, such mutagenesis screening is performed in the suboptimal host *S. cerevisiae* (Bulter et al. 2003, Alcalde et al. 2005, 2006, Madzak et al. 2006).

The *C. cinerea* laccase expression system

C. cinerea strain FA2222 is a strain with no laccase activity (Kilaru et al. 2006b) and has therefore been chosen as a production strain of individual enzymes. For efficient protein production, different promoters were tested in combination with *C. cinerea* laccase gene *lcc1* (Fig. 6A). The heterologous *Agaricus bisporus gpdII* promoter from a constitutively expressed glyceraldehyde 3-phosphate dehydrogenase gene (Kilaru & Kües 2005) was found to give highest enzyme activity (Kilaru et al. 2006b; Fig. 6). Transformed DNA integrates randomly into the genome (Granado et al. 1997). In consequence, transformants differ in quality since flanking DNA sequences can influence expression levels of inserted genes. Screening of a higher number of transformants is thus advisable. However, the coloured halos formed around colonies on ABTS-supplemented agar when expressing laccase (Fig. 6B) is an excellent pre-selection for high producers (Kilaru et al. 2006b). As expected, glycosylation of recombinantly produced protein Lcc1 was indistinguishable from the protein when produced in *C. cinerea* from its natural promoter (P.J. Hoegger unpublished).

Also the other 16 laccase genes of *C. cinerea* were transformed under the control of the *A. bisporus gpdII* promoter and at least five of these can give an active protein in *C. cinerea* but at different yields (Kilaru 2006). As far as yet known from protein analysis, the *C. cinerea* laccases differ in substrate spectrum and other enzymatic properties – enzymes with a pH optimum around pH 7.0 are of special interest (Saathoff 2005, Kilaru 2006, M. Rühl et al. unpublished). Ongoing work has proven that also laccase genes from foreign species can be expressed in *C. cinerea* (Grimrath 2007, P.J. Hoegger et al. unpublished). Since principally working, a research focus now aims on establishing best production conditions for transformants in fermenters (M. Rühl, unpublished).

Conclusions

More and more, fungal enzymes find applications in environment-friendly technologies in modern pulp and paper and wood industries as well as in ethanol production from lignocellulosic residues (Hüttermann et al. 2001, Kenealy & Jeffries 2003, Tengerdy & Szakacs 2003, Mai et al. 2004, Gray 2007, Jeffries 2006; see also Chapter 18 of this book). To make such processes economical, high quality enzymes in sufficient amounts at reasonable prices are required. Cellulases, xylanases, laccases, and other enzymes from different organisms and with different properties are commercially available, in some instances purified, more often only partially purified or crude and, therefore, they are possibly mixed with other enzymes and proteins. Often, the non-purified enzymes are sufficient for applications, as for example in wood composite production and in pulp bleaching. Active research in enzyme screening, optimising fermentation (SmF and SSF) and recombinant production is nevertheless ongoing to provide larger catalogues of easily produced enzymes with new and optimal properties as required for the various

industrial applications. Currently, lignocellulose attacking enzymes are produced predominantly by SmF, often with wildtype or mutant strains of natural enzyme producers, sometimes with recombinant strains. SSF processes, well established in Eastern cultures, now also receive higher attention in Western countries. SSF can be a particular good choice for enzyme production with fungi growing on solid lignocellulosic waste. Other promising yet not fully implemented approaches are those where different organisms are coupled in production and approaches where enzyme production and utilisation is combined. Establishing well-working recombinant production systems has the advantage that enzymes naturally produced in low amounts should become available at larger quantities. Moreover for recombinant production, enzyme properties might be optimised through genetic engineering. Using one or a few organisms in recombinant productions should furthermore eliminate the elaborate work to establish for every new natural producer and each of its interesting enzymes an own fermentation process.

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20. Recycling of Wood Composites and Solid Wood Products

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Introduction

Timber materials such as particleboards, medium density fibreboards (MDF), plywood, oriented strand boards (OSB), and other wood composites (see Chapter 15 of this book for descriptions of wood composites) are manifold applied in the production of furniture, panelling and roof covers, and other matters. Especially furniture has become a fashion commodity with continuously declining life-cycles. About 8 to 10 million tons of wood wastes accumulate in Germany per year, of which over 2 million tons are used furniture (Sundermann et al. 1999, Marutzky 2000). Other sources estimate the yearly disposal of used wood in Germany between 10 and 15 million tons (Harms & Flamme 1999). According to the German Technical Instruction on Municipal Waste (TASI 1993), starting with year 2005,



Fig. 1 Rejections of particleboards (left photo) and a heap of board material from discharged furniture and production remainders (right photo) as material for recycling in the panelboard industry

waste containing more than 5% of organic material cannot longer be deposited in landfills. Definitely, this applies to all timber products including furniture, production remainders and production rejections (Fig. 1). The Act for Promoting Closed Substance Cycle Waste Management and Ensuring Environmentally Compatible Waste Disposal from 1996 (Kreislaufwirtschafts- und Abfallgesetz – KrW-/Abfg; <http://bundesrecht.juris.de/bundesrecht/krw-abfg/gesamt.pdf>) specifies the legal regulations for recycling processes (von Köller 1996). The most environmentally benign utilisation - typically reuse of the material - shall be preferred. As an alternative to reuse the material, thermal utilisation is to be considered (Marutzky 2006). Combustion of discarded timber products for energy production is therefore a possibility - another is to disintegrate discarded wooden items for reuse of the wooden material for new products. Life Cycle Assessment (LCA) as defined in ISO 14 041 (ISO 1998; for special discussion of wood-based products see Jungmeier et al. 2002a,b, 2003, Werner et al. 2007) of two scenarios validates the recycling of wood waste for panelboard manufacture to be more favourable under an environmental perspective than pure combustion for energy production (Rivela et al. 2006a,b). Most effective is probably a combination of both, material reuse and energy production from the unusable wood-based waste in a cogeneration unit that generates energy for use in disintegration of old and production of new panelboards (Kirchner & Kharazipour 2002a, Smith 2004, Marutzky 2006), although geographical conditions of production sites have an impact on the overall energy situation (Rivela et al. 2007).

Types of wood waste material

Used wood for recycling comes from different sources such as households and recycling centres, building trades, and other industries. Depending on the source,

the types of wood waste material differ in percent. Of the 52 to 65% weight of wood-containing household waste in Germany, 7 to 13% are solid wood and 27 to 45% derived timber products. 33 to 42% of the waste from recycling centres present solid wood and 50 to 62% derived timber products. In contrast, over 50% of wood waste from building trade and other industries is solid wood. Most of the rest are derived timber products. Most importantly, the parts of treated and of coated wood and wood products differ with the source they come from. Larger amounts of untreated wood can be found in the solid wood waste fraction from building trades (between 20 and 56%) and other industries (about 15%). Derived timber products from households and recycle centres are most often coated whereas considerable parts of the wastes of the building trades (up to 30%) and industrial waste (slightly more than 10%) can be without. Impurities such as coatings, wood preservatives, binding agents and flame proofing agents determine the fate of wood and wood composites in recycling (Harms & Flamme 1999). Accordingly, waste wood is categorised for material recycling and thermal recycling in four classes (A I to A IV), to be found in the attachments of the German Waste Wood Ordinance (AltholzV 2002). Pallets and boxes and cases from solid wood, untreated solid wood from the building trade, solid untreated furniture from waste wood class A I as well as pallets, boxes, cases, wooden material from construction sites, furniture made from derived timber, used solid floor, ceiling and other boards from interior use, doors and door frames falling in waste wood class A II (free off organic halogen-compounds and other harmful substances) are allowed to be converted into wood chips for wood composite production. Certain waste material of class A III (e.g. furniture coated and painted with halogenated organic compounds, mixed waste wood from bulky refuse) can be also used in material recycling but only after prior removal of coatings and varnishes or when these were eliminated during processing procedures. In contrast, material falling into class A IV [e.g. wood with preservatives and other wood with high pollutant content, except if containing polychlorinated biphenyls (PCB)] can only be used in energetic recycling. Hazardous PCB-containing wood waste is to be specially treated according to the PCB/PCT (polychlorinated terphenyl) Waste Ordinance.

Disintegration of wood composites

Wood composites are products manufactured on the basis of mechanically chopped, milled and grinded (and refined) woods (veneers, strands, particles, fibres, etc.) bonded by adhesives (see Chapters 15, 16 and 18 of this book). For substantial recycling of wood composites such as particle boards from discharged furniture, production remainders and rejections (Fig. 1), the material needs to be disintegrated into small pieces. Three different principles can be applied for disintegration of panelboards: mechanical, thermo-hydrolytic and chemical, and combinations thereof (Fig. 2). Target of any process is to harvest the highest

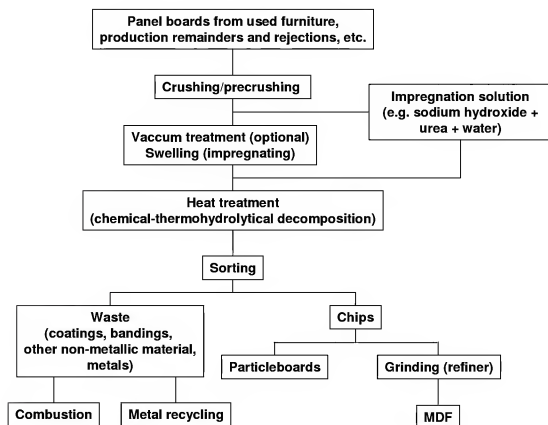


Fig. 2 Principle steps in the process of disintegration of panelboards for recycling in the wood composite industry. In mechanical disintegration, the waste material is chopped into small pieces that may go directly e.g. into new particleboard production. In thermo-hydrolytic disintegration; upon pre-crushing the material, heat, pressure and humidity are used to release the undamaged wood particles by dissolving the old resin. In chemical disintegration, chemicals are added to ease adhesive degradation (modified from Kirchner 2000; see text for further explanations)

possible amount of high quality recycling material for subsequent reuse e.g. in the panelboard industry.

Mechanical disintegration

Mechanical disintegration is always a dry process since performed in absence of water. In mechanical disintegration, discharged wood composites are crushed into small pieces without resolving the overall texture of the derived timber material. Dry chips obtained from mechanical crushing might directly be used in a new application. However, chips from mechanical disintegration of wood composites have usually a cubic shape (Fig. 3), contrary to primary wood chips that are more flat. This cubic shape is of disadvantage for later particle board production, since



Fig. 3 Wood chips obtained from mechanical disintegration of particleboards; photo kindly supplied by M. Rühl (size bar = 1 cm)

it negatively influences board properties (Nonninger et al. 1997; A. Kharazipour, unpublished observations). Typically, the structure of wood fibres in the chips resulting from mechanical disintegration is to a high degree damaged. Loads of dust and fine particles are generated during the crushing. Dust and fine particles are lost material and have to be separated from the utilisable chips for thermal recycling (Roffael et al. 1996). Moreover, chips from mechanical disintegration have a lower water retention value (WER-value) and the wettability with UF- (urea-formaldehyde) resins, PF- (phenol-formaldehyde) resins and binders based on PMDI (polymers of diphenyl-methandiisocyanates) tends to be poorer compared to fresh chips and to chips obtained from thermo-hydrolytic degradation of panelboards (Roffael & Schneider 1978, 1979, Roffael et al. 2003, Hameed et al. 2005a,b; for details on conventional binders see Chapter 15 of this book). A positive effect from dry mechanical disintegration is that no drying processes for the chopped material are needed which saves energy and avoids emissions that would otherwise be generated during a drying process. Main inventions making use of mechanical disintegration are shortly presented in the following.

In the **“Retro-Verfahren” (retro-process)** invented by Roffael et al. (1996), panelboards with hydrolysable and formaldehyde-containing adhesives are crushed into so-called **“retro-chips”**. Upon size-selection for outer and inner layers of new particle boards, retro-chips will be mixed with tannins as a natural binder (see Chapter 16 of this book) and blended in amounts of 10-15% with UF-coated fresh wood chips for pressing into new boards. Pressure, humidity and temperature hydrolyse the hardened old binder on the retro-chips which in turn will condensate with the tannins to a tannin-formaldehyde resin. Consequently, the retro-process reuses both, the wood chips and the binder from the old boards (Kharazipour & Roffael 1997a).

The “**Retro-Amino-Verfahren**” (**retro-amino-process**) is a technical advancement of the earlier retro-process that makes use of disposed aminoplast-containing composite wood in particle board production. Both coated and uncoated material may be recycled in totality. To retro-chips of defined size (about 0.5–35 mm in length for the middle layer of particle boards and 0.1–2 mm in length for the overlays) in amounts of 1 to 20% of their weight (optimum 5–6%), an amino-group-containing compound is added in order to bind free formaldehyde. This can be e.g. urea in a watery solution for best wetting. Upon merging with aminoplast resin-coated virgin chips, the resulting chip mixture is pressed at high temperature into panelboards. Old bonds in the recycled chips are resolved and, thereby, formaldehyde is released to consecutively react with the added amino-group containing substance. An aminoplast resin results that binds the chips in the newly produced boards (Kharazipour & Nonninger 1997b).

A special development of mechanical disintegration is presented by the **REHOLZ-process**. Using a special patented cut-off device in this process, panelboards are sliced into small flat discs being in average 2 mm thick, 15 mm wide and about 40 mm long. Following separation of metallic and inorganic contaminations and size fractioning, such discs might be further handled as oriented strands. Boards produced from such chips have however an uneven surface due to the relative large size of the discs and the incomplete separation of coatings. Therefore, the boards are of good value only for the building industry (Möller 1994, Möller & Herrlich 1994, Michanickl 1996, Möller & Förster 1997).

Thermo-hydrolytic disintegration

In thermo-hydrolytic disintegration, steam and pressure are used to cleave existing bonds in wood composites that were glued by hydrolysable adhesives. By various economical reasons (such as low material price, short pressing times, a translucent colour), in most instances this will be UF-resins (see Chapters 15 and 16 of this book). In contrast, panelboards with melamin-modified UF-resins, PMDI and phenol resins cannot be recycled through thermo-hydrolytic processes since these binders are hydrolysis-resistant (Franke & Roffael 1998, Kirchner 1997, 2000).

In hydrolytic dissolution of a hardened UF-resin, fragmentation of the polymer occurs by splitting internal methylene-ether bonds and methylene-bridges. Hydroxy-methyl-groups are generated from which formaldehyde can be released. If not removed from the system, the formaldehyde can react by additions again with the urea-derivatives resulting from the dissociation of the formaldehyde from the hydroxy-methyl-groups (Franke & Roffael 1998, Fleischer & Marutzky 2000). Various parameters determine the degree of hydrolysis of hardened UF-resin. Of these, a high temperature is most decisive (Wittmann 1962, Stöger 1965, Ginzl 1971, 1973, Raue 1971, Myers 1982, Franke & Roffael 1998, Fleischer & Marutzky 2000, Roffael & Kraft 2004). In consequence, thermo-hydrolytic disintegration of



Fig. 4 The thermo-hydrolytic Conti-Recycling-Process developed by the Pfeleiderer Holzwerkstoffe & Co. KG for disintegration of particle boards (Kharazipour et al. 1999, 2000, Kirchner & Kharazipour 2002a,b, Stracke et al. 2001). Wood composites are loaded into a conventional pre-crusher (shown in full view in the upper left photo; loading is shown in the upper right photo). Palm-sized pieces from mechanical chopping in the pre-crusher (see Fig. 5) are subjected in screw conveyors in a disintegration plant (lower left photo: pilot plant at Rheda, Germany) to decomposition in a closed system of saturated vapour typically at a temperature between 120 and 180°C (optimum 140°C). Sorting of the chip material by size into cover and intermediate layer chips for particle production and separation from oversized wood chips, coating remainders and other foreign matter is performed under vibration in a terminal sieving step in a vibro-sieve chamfer-line (shown enlarged in the lower right photo). For further details see Fig. 6

panelboards is performed under pressure at a temperature range between 120 and 180°C (Anonymous 1992, 1993, Kharazipour et al. 1999, 2000, Stracke et al. 2001), respectively between 110 and 150°C (Fleischer & Marutzky 2000). Times of heat treatment are determined by the size of the wood composite pieces to be dissolved and by the moisture expansion of the material. Pre-crushing of panelboards (Fig. 4 and 5) and swelling of the resulting pieces in water or by steam application are therefore integral pre-treatments in thermo-hydrolytic disintegration



Fig. 5 Palm-sized pieces (approximately 5-10 x 10-20 cm) of particle boards (upper photo) as obtained from pre-crushing in the thermo-hydrolytic Conti-Recycling-Process of the Pfeleiderer Holzwerkstoffe & Co. KG (compare Fig. 4 and 6). Upon thermo-hydrolytic disintegration, sieving separates chips of intended sizes of 0.1 to 20 mm (lower panel right) from those that are too large and from other contaminations (upper panel left)

processes (Fig. 2 and 6). Due to the pre-crushing, only a small part of chips resulting from thermo-hydrolytic disintegration will be of cubic shape whereas the majority of the released chips will retain the shape (see Fig. 5, bottom right) and therefore the quality of the original chips (Kharazipour 2004). However, remains of resins may still adhere on the chips (Marutzky 1994, Kirchner 2000) that will give rise to formaldehyde emissions during subsequent chip drying (Wagner & Roffael 1996). The extra energy costs for the drying present a further downside of the wetted chips.

Historically, the **Sandberg-process** was the first thermo-hydrolytic process that came into action in particle board production companies for in-house recycling of production remainders with turnover rates of 25 tons of chips per day. The process used steam at a pressure of 1 to 5 bar to disintegrate in 0.5 to 4 h bonded wood chips. With 10% water content, chips could directly be applied in new productions in percentages of up to 30% of the total raw material. However, the long steaming times were uneconomical why over the time the process has been dismissed (Schlippak 1965).

The **Pfeleiderer process** developed and patented as a batch process in 1992 (Anonymous 1992) and subsequently as a continuous process (Kharazipour et al. 1999, 2000, Stracke et al. 2001; Fig. 4 and 6) considers UF-containing particle boards and MDF from used furniture as well as any production remainders and rejections, regardless of whether coated or not. This mixed material has to be bro-

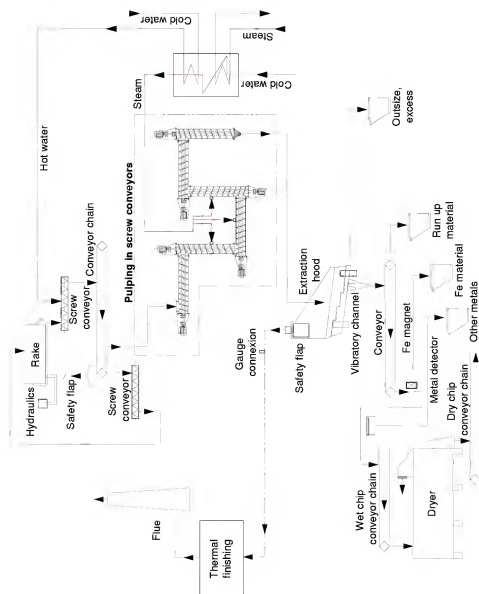


Fig. 6 Scheme of the thermo-hydrolytic Conti-Recycling-Process developed by the Pfeleiderer Holzwerkstoffe & Co. KG for disintegration of particle boards (Kharazipour et al. 1999, 2000, Kirchner & Kharazipour 2002a,b, Stracke et al. 2001)

ken in conventional crushers into pieces of an approximate size of at least 5-10 cm x 5-10 cm (Fig. 5). A significant amount of foreign materials, such as metals and non-metallic waste parts, is separated from the board fragments by a magnet and a non-metal separator, respectively. The wood-containing pieces left are soaked for about 10 min at a temperature of 90 to 95  C in water for uptake of a defined amount of water (70-80% of the weight of the air-dry material). This pre-swelling needs to be even over the whole material for best efficiency in the following thermo-hydrolytic disintegration and depends on the water temperature, time of soaking, the sizes and gross density of the board pieces, and the relative amounts of coated and uncoated pieces. Upon transfer into a pressure chamber, the binder disintegrates within 10 min by treatments with steam at pressures of maximum 3 to 6 bar (optimum 4 bar). The neatly dissociated, largely intact chips might afterwards be cleaned from adhesive remainders and other impurities by a stream of steam that hydrolyses the left resins. Fractioning by drum- or vibro-sieves separates chips of different sizes and removes any coating remainders, metal and plastic pieces, etc., that still resided in the material. Laboratory tests showed that up to 100% of wood chips recycled by the Pfeiderer process can be used in production of new particle boards (Kirchner 2000).

The **Pfeiderer batch process** is analogous to autoclaving (pressure-cooking) and is performed in cycles of loading of material into a pressure-resistant vessel, pressure treatment with steam, evacuation of steam and emptying the material from the vessels. The whole retention time within the vessel takes about 1 to 2 h (Anonymous 1992, 1993). Emissions (formaldehyde, etc.) can be a problem during steam evacuating and re-opening the vessel. In contrast, in the continuous thermo-hydrolytic disintegration process, the **Conti-Recycling-Process** (Fig. 4 and 6), the retention times of the material are much shorter (about 5 to 7 min) and emissions are continuously evacuated for direct fume combustion. The whole process is performed in a closed system in which the retrieved chip material is fed into the likewise continuous particle board production (Kirchner & Kharazipour 2002a,b). The Conti-Recycling-Process became possible by the invention of a system of five and more serially arranged, in line-operating spirals which act in the successive process of panelboard disintegration (Fig. 6). By steam pressure (between 2 to 6 bar), aminoplast-bonded material is hydrolysed at temperatures between 120 to 180  C (optimum 140  C) and pushed through the screw conveyors towards the respective frontal outlet which is flanged to the inlet of the next spiral in such way that the temperature and pressure parameters maintain constant over the whole transport process. The outlet of the last screw conveyor ends on a vibrator channel for material sorting through sieving. Chips fall through the vibrating sieve onto a conveyor and are then transported into a dryer, whilst the excess (metal pieces, coatings, larger wood pieces, non-disintegrated wood composites) is transported into a waste container and the formaldehyde released during hydrolysis of the material is removed through a safety flap in order to be burned. On its

way into the dryer, small iron peaces are separated from the bulk chip material by magnets and other metals by a different flow behaviour compared to wood chips when ejected from the conveyor. The pure wood particles are still hot with temperatures above 100°C when arriving in the dryer. These high temperatures save energy in the particle drying. The dried material can go directly into particle board production (Kharazipour et al. 1999, 2000).

An analogous development to the Pfeiderer batch process is the **WKI-process** of the Wilhelm-Klauditz-Institute in Braunschweig (Michanickl & Boehme 1995a,b). This batch process bases also on pre-crushing of rejected panelboards and furniture with subsequent steam-induced dissociation of hydrolysable adhesives. Differently to the Pfeiderer process, the WKI process uses larger pieces of board material (10-20 cm x 10-20 cm) that for about 30 min are subjected to a swelling process in an impregnation solution (tempered 90°C) in a pressure tank in which they take up the solution in amounts of up to 70% of their net weight. Impregnation is enhanced when vacuum (about 200 mbar) is applied before filling the vessel with the liquid. Upon completed impregnation and pumping down the remaining solution, board pieces are heated to a temperature of 80 to 120°C for disintegrating the resins. At a temperature of 110°C, this should take about 20 min. Together with the time required for impregnation, the total time to complete the process takes longer than 60 min. Wood chips recovered upon opening the reactor at the end of the process need also to be dried. Because they are cooled down to room temperature to safely open the vessel, this costs more energy compared to the drying in the Conti-Recycling-Process described above.

In 1996, the **Formaplan-Process** (FORMAPLAN Holzwerkstoffe GmbH & Co.) was designed for the transfer of the WKI-process to an industrial application of thermo-hydrolytic disintegration of panelboards glued by hydrolysable adhesives and/or by resins apt to chemical decomposition. In this industrial up-scaled process, broken material of a size smaller than 2 cm was separated after pre-crushing from larger pieces (up to 10 to 20 cm) in order to transfer these directly to new particle board production, reducing the total volume of material for thermo-hydrolytic disintegration by up to 10%. Larger pieces were soaked under negative pressure (500 to 700 mbar) for 15 min in an impregnation solution of a temperature of 75-90°C which doubled their weight. Upon release of the surplus solution, the swollen board pieces were steam-treated for 40 to 70 min at 120 to 140°C and a pressure of 1.9 to 3.6 bars. Further 5 to 10 min were needed to siphon condensate and steam. Together with loading and emptying the vessels, the whole thermo-hydrolytic disintegration step required 2 hours (Stein 1998).

In 1997, also the company Nolte GmbH & Co. KG (Germersheim, Germany) installed the WKI-Process on industrial scale in a plant with an annual production of 55,000 t of recycled particle boards. Up to 50% of the whole chip load processed by the company per year in wood composite production might principally

be replaced by recycled material from this plant in a self-sustainable manner (Wittke 1998). Currently, this company is the only one in Germany producing recycled chips. Its actual yearly production rate has been slightly reduced to 30,000 to 35,000 t (Marutzky 2006).

Disintegration by chemical pulping

Roffael & Dix (1993, 1995) presented a chemical-thermal process that is applicable for both, hydrolysable resins as well as for non-hydrolysable resins. Thus, the most important advantage of this process over others is that panelboards glued with adhesives such as PMDI and PF can be managed for material recycling, although under harsh conditions. Unlike in all other processes, the morphological structure of wood will however be disrupted by the chemical-thermal process. Since the result of this process is a pulp, not only particle boards but also fibreboards can be treated.

As in the previously described processes, also at the beginning of the chemical-thermal process waste woods will be freed of metals, plastic etc. and chipped into small pieces. Comparable to conventional chemical pulping, these chips are treated under pressure with caustic sodium hydroxide (**alkaline pulping**) or a mixture of sodium hydroxide and sodium sulphite (**alkali-sulphite pulping**). The fibres in the wood chips are turned into a chemical pulp and the binders as well as any additives can be collected in the spent liquor (Roffael & Dix 1993, 1995). The fibre material might be further used in paper making or in fibreboard production (Dix et al. 2001a,b), although it is dark in colour and not as attractive as other pulps. The resins after concentration need either to be burned or, alternatively, they might be used as an extender for UF- and PF-resins (Roffael 1997). However, since coated and painted material among uncoated particle boards are treated in this process it can happen that toxic compounds are generated during decomposition that will accumulate in the spent liquor (Michanickl 1996).

Material recycling in panelboard production

Material reuse from discarded timber products doubled in Germany in the last decade (Sundermann et al. 1999, Kirchner & Kharazipour 2002b, EPF 2005). In 1995, 10% of the total raw material in particle board production was recycled wood (Harms & Flamme 1999). According to the European Panel Federation (EPF 2005), in 2003 19% of the wood material in the German and 23% in the European particle board production came from recycled wood. Best values in the range of 25% of recycled wood have been accomplished in Belgium, Denmark, and the UK (EPF 2005). In Germany, a total of 9,312,000 tons of particle wood was produced in the year 2003 (FAOSTAT 2007; see Fig. 3 in Chapter 15 of this book), i.e. nearly 1.8 million tons of wood material (sawdust, shavings, veneer, chips etc.) were recycled in particle boards.

Chips recovered from particle boards by dry mechanical disintegration tend to be of poorer quality compared to those obtained from virgin wood from the forests (Nonninger et al. 1997). For manufacturing high-quality particle boards under conventional production conditions, no more than 20% of the raw material should come from such recycled wood. Indeed, this corresponds to the current amount of recycled wood in the German particle board production. Usually, the particle board industry adds at least up to 7% of material recycled from own rejections to their productions but in some plants values up to 35% are reached (Marutzky 2006). Higher amounts of the more or less cubic-shaped chips from dry mechanical disintegration negatively affect the mechanical-technical properties of the produced boards (Kharazipour & Nonninger 1997a, Kharazipour & Roffael 1997). In addition, due to the lower wettability of the recycled chips, higher amounts of adhesives are required for gluing and the formaldehyde release from the products is thus enhanced (Hameed et al. 2005b). This is less true in the retro-process in which recycled chips are tannin-coated and where up to 15% of tannin-coated retro-chips might be used without loss of board qualities (Kharazipour & Roffael 1997). However, tannin is a very costly binder (see Chapter 16 of this book). In the retro-amino-process, urea-solutions are used to coat the recycled chips and a maximum of 50% of recycled chips material might be applied without an effect on board properties (Kharazipour & Nonninger 1997a).

Particle boards with 100% waste wood have been produced for the market in Italy (Roffael 1997, Marutzky 2006). If chips are obtained from wood composites by thermo-hydrolytic disintegration, particle boards newly produced from 100% waste wood can have the same or even better properties than the former generation of boards (Michanickl 1996, Kirchner & Kharazipour 2002a,b). Particle board production and thermo-hydrolytic disintegration can be performed repeatedly with the same chips without losing the required properties of boards produced from the recycled material (Michanickl 1996, Kirchner 2000).

Hameed et al. (2005a) report that chips from thermo-hydrolytic recycling of particle boards by the WKI-process can be more hydrophilic than primary chips, possibly due to hydrolysis of the resin and a partial elimination of acetyl groups from the wood during the thermo-hydrolysis. Michanickl & Boehme (1995a,b) and Kirchner & Kharazipour (2000a,b) tested particle boards from 100% thermo-hydrolytic recycled material and from such material mixed with virgin chips. Swelling properties in boards made from recycled material was reduced compared to boards made from 100% virgin chips.

Likewise, MDF made from mixtures of fresh fibres and fibre material obtained from chemical-thermal disintegration of OSB had a decreased thickness swelling compared to MDF made only from fresh fibres (Schoo et al. 2003). According to Schoo et al. (2003), possible reasons for this are that resins and agents used for hydrophobation in OSB production remained still attached to the fibres and that the content of hydrophilic hemicelluloses had been reduced by the chemo-

thermo-mechanical treatment. Furthermore, addition of recycled fibre material lead to an increase in the internal bond strength but unfortunately also to a decrease in bending strength (Schoo et al. 2003). MDF made from chemical pulp of waste particle- and fibreboards met the standards defined for MDF, although bending strength and internal bond strength was not as good as in MDF made from virgin fibres. In contrast, thickness swelling was clearly lower in MDF with the recycled material and, another important aspect, the boards had a lower content of hazardous formaldehyde (Dix et al. 2001a,b).

Risk assessment of wood material

Contamination of wood wastes with preservatives and other pollutants limits opportunities for their recycling in panelboard production. Upper limits of pollutant concentrations in recycled wood are defined in Germany by RAL GZ-428 (RAL 2003). Knowledge on the kind and conditions of usage and the age of wooden products can only be a rough indication on what types of pollutants might be expected to reside in the material. For precise identification, techniques for reliable, quick, specific and sensitive assessment of potential pollutants within wood waste materials are required and in many instances yet to be established. Organic compounds might be detected by complex gas chromatography/mass-spectrometry (GC/MS) and ion-mobility spectrometry (IMS) (Peek 1998, Peylo & Peek 1998, Schröder et al. 1998). Sensitive tests for detection for example of polychlorinated biphenyls and of dioxins and dioxin-like compounds have recently been presented, using GC/MS (Schulze et al. 2003) and the biological CALUX luciferase test [Asari et al. (2004); see Chapter 12 of this book for further explanation], respectively. Other newer developments concentrate on sophisticated techniques for sorting the contaminated material from acceptable wood waste. X-ray fluorescence spectroscopy (XFR) and laser-induced breakdown spectroscopy (LIBS) are promising detector technologies to identify CCA (chromated copper-arsenate)-treated wood and CCA-containing paints on wood, and also other types of preservatives (Peek 1998, Peylo & Peek 1998, Uhl et al. 2001, Blassino et al. 2002, Moskal & Hahn 2002, Solo-Gabriele et al. 2004, Martin et al. 2005, Block et al. 2007, Dubey et al. 2007).

Recycling of polluted material in panelboard production

During the last two decades, CCA has been the primary preservative in the USA. Each year, over 14.2 million m³ CCA-treated lumber is produced with an average estimated service life of 25 years (Micklewright 1998). Vast volumes of CCA-treated wood products (estimate for the year 2020: 16 million m³) are expected to come out of service during the next decade why there are urgent needs to find suitable recycling concepts (Fulton & DeGroot 1996, Kabir et al. 2006). Tests in the USA showed that CCA-treated wood chopped into chips and flakes could poten-

tially be used in particle- and flakeboard production, if required also after (partial) remediation of the toxic metals (Munson & Kamdem 1998, Mengeloglu & Gardner 2000, Clausen et al. 2001, 2006, Kartal & Clausen 2001, Li et al. 2004a,b). The best ways of remediation for wood chips and flakes for recycling in particle- and flakeboard production are still under research. In such processes, the overall structure of the wood must be preserved as best as possible. CCA-remediation of wood can be done e.g. by oxalic acid extraction, microbial treatment, or combinations thereof. Microbes functioning in bioremediation of CCA-treated wood wastes include specific bacteria, wood-inhabiting stain fungi, molds, and brown rot basidiomycetes that produce sufficient amounts of oxalic acids (Clausen & Smith 1998, Clausen 2000, 2004, Kartal et al. 2004, 2006).

So far, only material treated purely with oxalic acid and material treated with oxalic acid and the bacterium *Bacillus licheniformis* have been tested in panelboard production. The removal of toxic metals from CCA-treated wood was however in neither case complete. Particle boards made from such partially purified chips had reduced internal bond strength and an increased swelling thickness. Most interestingly, flakeboards made from purified material had properties alike to flakeboards made from untreated material. Flake geometry and type of resin, as well as differences in surface area and metal removal compared to wood chips are discussed as possible reasons for the generally better results of the flakeboards in mechanical and physical performance compared to the particle boards (Clausen et al. 2001, 2006). A study on flakeboards produced from non-remediated CCA-treated chips supports these conclusions. Performance of flakeboards from CCA-treated wood-chips was seen influenced on the one hand by the shape of the flakes and on the other hand by the type of binder used for gluing: disk flakes performed better than ring flakes and PF resin better than PMDI. Nevertheless, in the study the overall performance of the flakeboards from CCA-treated wood was always inferior compared to flakeboards from untreated wood (Mengeloglu & Gardner 2000).

CCA still residing in chips and flakes after oxalic acid extraction and after bioremediation (17 to 20% copper, 14 to 29% chromate, 5 to 11% arsenate) gave a better fungal resistance to the panelboards, although the tested particle boards showed generally high leaching losses of the remaining elements (Clausen et al. 2001, Kartal & Clausen 2001). Munson & Kamdem (1998) proposed to make best use of the CCA-polluted material for further protection of derived products for exterior use. Mechanical and physical properties of boards as well as decay resistance are acceptable if the fraction of untreated CCA-containing wood flakes and chips goes not above 50% (Munson & Kamdem 1998, Li et al. 2004b). A problem to solve is the poor performance of adhesives with CCA-treated wood (Mengeloglu & Gardner 2000, Lee et al. 2006). Another is certainly the leaching, that occurs with panelboards made from CCA-containing wood material. The higher the amount used in mixtures with fresh material, the higher is the leaching (Kartal &

Clausen 2001, Li et al. 2004a). This latter problem addresses the problem of the environmental load caused through usage of the toxic preservative CCA. In Germany, CCA-treated wood is not anymore allowed (see Chapter 13 of this book) and production not even of partially CCA-cleaned boards can be an option (Alt-holzV 2002). In the USA, where usage of CCA is still common praxis, reduction of the amounts of required new loads of CCA for outdoor wood protection are environmental arguments expressed for developing options for recycling CCA-treated wood as a material (Munson & Kamdem 1998). A survey amongst manufacturers in 1998 revealed however that, at least at that time, they were not much in favour of using such wood in conventional wood composite production by concerns for the safety of workers and environmental problems (Smith & Shiau 1998). Application in wood-non wood composites was suggested and research is active also in such directions (Zhou & Kamdem 2002, Kamdem et al. 2004).

Another problematical group of waste woods that should be mentioned are discarded railway sleepers and other poles treated with creosote (Fulton & DeGroot 1996). By the high contents of carcinogenic polycyclic aromatic hydrocarbons (PAHs) in creosote (see Chapter 13 of this book), in Germany they cannot be used anymore as constructive element in parks and gardens (<http://217.160.60.235/BGBL/bgb11f/bgb1102s3185.pdf>), although in other countries this is still very popular (Ikarashi et al. 2005, Werner et al. 2007). In Northern America for example, concerns on negative effects on health and environment are less developed, but reservations against application of treated wood in outdoor applications such as in childrens' playground equipment are increasing (Vlosky & Shupe 2004a,b, 2005). Application of creosote-impregnated wood in decay-resistant wood composites for outdoor use had been suggested (Roliadi et al. 2003, Gardner et al. 2003), but newer research targets at removal of creosote from the material by hydrothermal treatments (Catallo & Shupe 2003, Shupe et al. 2006). Efficiencies of such treatments are in the range of 98-99% of creosote removal (Catallo & Shupe 2003, Shupe et al. 2006). Further bio-polishing with microbial consortia (such as mixtures of strains from the bacterial genera *Pseudomonas*, *Flavobacterium* and *Acinetobacter* strains) is possible (Portier et al. 1996). All in all, taking in opportunity-costs such as for purification and other processing, incineration with co-generation of energy is however an easier and more eco-friendly solution for decommissioned creosote-containing wood (Werner et al. 2007).

Other types of material recycling

The technical requirements for waste wood processing, for possible purification from pollutants, and for quality control of wood waste for material recycling are extra cost factors in panelboard production. With rising prices and reduced supply of raw wood from forests, recycling of wood waste is nevertheless recognised an economically attractive alternative - in addition to that the material reuse brings



Fig. 7 Recycling of wood chips obtained from disintegration of particle boards in mushroom production. Left photo: a view into a mushroom bunker with bags filled with such chips for production of fruiting bodies of *Pleurotus ostreatus* (oyster mushroom); photo at the right: a block of wood chips fully colonised with *P. ostreatus* at the time of fruiting body maturation (further information in Chapter 22 of this book)

ecological benefits (Marutzky 2006). On the contrary, the recent promotion of biomass for production of energy and the installation of several technically advanced power stations for electricity and heat production (see Chapters 5 and 6 of this book) shortened the market of high-quality waste wood. Possible consequences are that less appropriate waste wood might be used in wood composite production, board qualities in turn decrease and pollutants might be shifted (Patzek & Pimentel 2005, Marutzky 2006). For higher inputs of less quality substance from waste wood in panelboard production, technical parameters have to be challenged for both, elimination of foreign matter and pollutants and for of generation of chips and fibres of better quality (Marutzky 2006).

Currently, particle board production has the largest share in reuse of wooden material (EPF 2005). Research has also demonstrated that fibres released from particle board, OSB, and MDF can be recycled in MDF production (Dix et al. 2001a,b, Schoo et al. 2003; see above). Furthermore, wood-fibre plastic compounds might be produced (WPC; Boeglin et al. 1997, Balatinecz & Sain 1998, Balasuriya et al. 2003), or wood cement composites and light-weight bricks (Stahl et al. 2002, Qi et al. 2006). With WPC, repeated material recycling has recently been tested. Composite properties remained stable over a few cycles of extrusion-milling but after 10 and more cycles flexural strength of the WPC material decreased (Augier et al. 2007). In such way, particularly inferior material from recycling found not anymore suitable for particle- and fibreboard production might still find repeated use.



Fig. 8 Briquette machine (full view at the top; top left: view into the dosing bin showing a rotating wheel pushing particles into the inlet of the briquette press; row below: enlarged views of the engine and the outlet of the ready briquettes) in the Biotechnikum (pilot plant station) at the Institute of Forest Botany in Göttingen used to fabricate briquettes from wood waste materials (bottom right; photos by S. Dantz & C. Schöpfer). Research projects on fabrication of briquettes address different lignocellulosic materials, different types of bonding agents, and heating values of resulting products (Vetter et al. 2007)

There are further options to use recycled wood chips and fibres as material. Applications encompass mulching material for soil covering and peat substitute for horticulture substrates (Chong 1999, 2005; see Chapter 24 of this book), structure material for sewage sludge composting (Lin et al. 2001), beddings e.g. in riding schools and in breeding of various animals (Hester et al. 1997, Anonymous 2000, Asari et al. 2004, Burn & Mason 2005), production of low grade paper (Roffael & Dix 1993), mushroom production (Kharazipour & Hüttermann 1997; Fig. 7), enzyme production in solid-state-fermentation (SSF) with fungi (see Chapter 19 of this book), and the production of briquettes (Demirbas et al. 2004, Vetter et al. 2007; Fig. 8) and charcoal (Bao et al. 2001). Also in all of these applications, care has to be taken on the quality of wood waste material and particularly on the presence of any contaminants and pollutants (Helsen & Van den Bulck 2005, Shibata et al. 2006).

Conclusions

Development of efficient economical and environmentally-friendly recycling processes is required to meet the increasing demands for wood-based products at times of wood shortage. Although there is active research, material recycling processes are yet little installed on industrial scale. Due to political measures, energy production from biomass is currently in the centre of attraction (see Chapter 5 and 6 of this book) and, by that, energetic recycling of wood very much outrivals the material recycling.

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Part VI – Other Commodities from Wood

21. Conversion of Biomass to Fodder for Ruminants or: How to Get Wood Edible?

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Introduction

At present, many developing countries are facing a vicious cycle: the increase in population is accompanied by a dramatic decrease in availability of arable land per capita (Fig. 1). The resulting problem of overgrazing is considered the main factor for desertification (Dregne 2002, Scheffer & Carpenter 2003, Herrmann & Hutchinson 2005; for North-America see e.g. Manzano & Nívar 2000, Manzano et al. 2000, for Africa e.g. Bencherifa 1996, Bouzid 1996, Johnson 1996, Miller 1996, Salihi 1996, Sutton & Zaimche 1996, Mazzucato & Niemeijer 2002). The concomitant degradation of fragile arid and semi-arid lands has a huge impact on the larger scale processes of global climate change (Potter et al. 1996, Vagen et al. 2005).

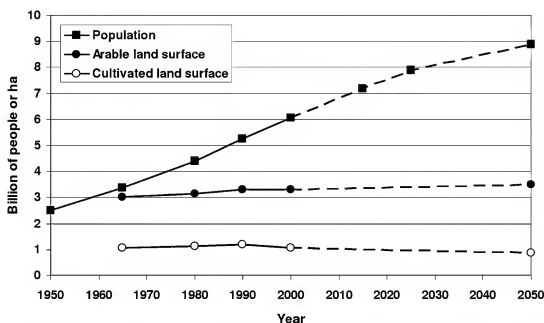


Fig. 1 Development of the population of the world and the available arable land on earth (Kitagawa & Yamamoto 2006); squares: world population in billion people, closed circles: total available arable land in billion ha, open circle: total cultivated land in billion ha, dashed lines: speculated development

The estimated limit of the world population with 7.7 billion people – calculated by available biotic and abiotic resources necessary for sustainment of reasonable living standards, production of food, energy and industrial products – will be reached already in the years 2025-2030 (Van den Bergh & Rietveld 2004; compare Fig. 1). It has to be expected that within a short time period the productivity of the available arable land will not be able to cope with the demand of the people for high value food (Bergström 2004). As Brown (1995) has convincingly shown, this will have a tremendous impact on the global trade and the political situation, with disastrous effects on the poorer countries of the world. Therefore, it is mandatory to exploit all possible resources for the production of high value food. A resource which would be available for food production without high efforts and environmental costs are the lignocellulosic wastes which accompany crop production, such as straw.

Lignocellulose and nutrition

The most important substance for human (and animal) nutrition is glucose. It exists in nature mainly as a polymer and the most important glucose polymer for animal (and human) nutrition is starch. Starch consists only of glucose molecules that are bound together in a very specific way, an $\alpha(1-4)$ -glycosidic bond as it is

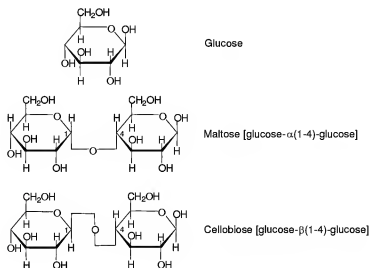


Fig. 2 Chemical formulas of glucose, maltose (the basic building block of starch), and cellobiose (the basic building block of cellulose)

the case in maltose (Fig. 2). Starch is the nutrient deposit in many seeds but it is not a very abundant molecule in nature. The most abundant natural glucose polymer is cellulose and the dominating organisms of the terrestrial biosphere, the trees, consist to about 40% of cellulose (Willför et al. 2005). Cellulose also is made entirely of glucose units. The difference to starch lies only in the stereochemistry of the linkages between the single glucose molecules. Linkages in the cellulose polymer are β(1-4)-glycosidic bonds. This is shown in the formula of cellobiose, the basic building block of cellulose (Fig. 2).

This for non-chemists apparently subtle distinction between an α(1-4)- and a β(1-4)-glycosidic binding between the glucose monomers of a macromolecule represents for animals the difference between life and death. A starch wafer which consists entirely of pure glucose monomers can be eaten and digested by humans and all animals. In contrast, humans and most animals are not able to survive by pure cellulose feeding although cellulose is an almost pure glucose polymer. The only mammals which can live on cellulose are the ruminants. They have very large stomachs with several chambers in which bacteria and ciliates live as symbionts. These micro-organisms are able to enzymatically hydrolyse cellulose (and hemicelluloses). The animals - goats, sheep and cows - chew several times the grasses which they take up and thereby disintegrate mechanically the plant material into small particles that are then susceptible for microbial degradation in the rumen (Wang & McAllister 2002, Kamra 2005). Glucose resulting from this degradation process is the basic nutrient for the host and the microbial symbionts. This symbiosis enables the ruminants to live on grasses which are indigestible for other

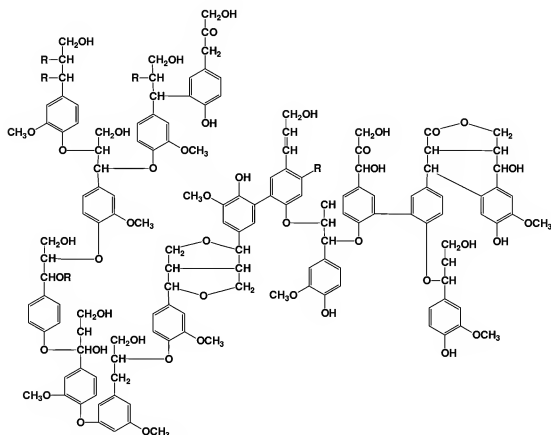


Fig. 3 Schematic formula of spruce lignin structure (adopted from Adler 1977)

animals and humans. However, ruminants are not able to live on wood or on plant parts which have a high content of woody cells (Akin 2007). Even wheat straw, consisting of up to 80% of carbohydrates, cannot be used as fodder for ruminants (Wang & McAllister 2002, Kamra 2005). The reason for this is the special aromatic substance built up from phenyl-propane units which converts plant tissues to wood: lignin (Fig. 3; see also Fig. 2 in Chapter 7 of this book).

In the woody cell wall, the lignin surrounds the carbohydrates – cellulose and hemicelluloses (Fig. 4). This protection is so efficient that hydrolytic enzymes from the rumen bacteria are not able to penetrate to the carbohydrates and degrade them into simple sugars. The only organisms which are able to break down the lignin are the wood degrading white-rot fungi (Fig. 5). Owing to special oxidative enzyme machineries, they are able to depolymerise lignin (Leonowicz et al. 1999; for further details see also Chapter 17 of this book) which is the first and most important step for the recycling of wood in the terrestrial carbon cycle. The term white-rot indicates that these fungi preferentially degrade the “brown” lignin moiety of the woody cell wall, thus enriching the “white” cellulose. The attack of wood by white-rot fungi is a process existing since the first lignin-containing plant

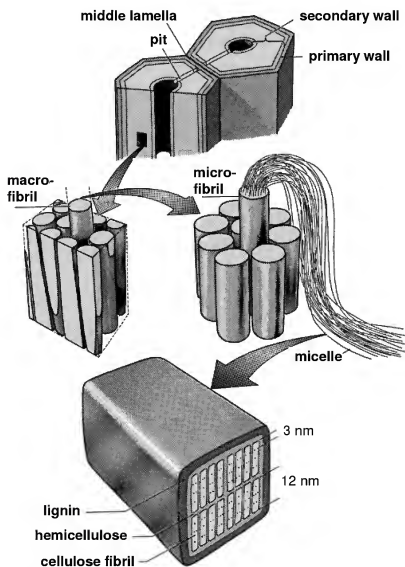


Fig. 4 Schematic view of a woody plant cell wall with: cross-section through a wood fibre shows the middle lamella and the cell wall with the primary and secondary cell walls, cross-section through a part of the secondary cell wall with macrofibrils, a bundle of microfibrils, the micelle strands, and cross-section through a micelle (after Hüttermann et al. 2001; drawn by G. Tambour, Faculty of Forest Sciences and Forest Ecology, Georg-August-University of Göttingen)

cell walls developed. Fig. 6 shows a segment of a cross-section of a petrified fossil palm from Brazil, where the outer layer has been almost completely delignified by a fungus before the process of petrifying was completed. The lignin-containing wood appears dark because of the heavy-metals which have been complexed by the lignin during the petrification. The periphery of the fossil trunk consists of



Fig. 5 Oyster fungus (or oyster mushroom, *Pleurotus ostreatus*), a white-rot fungus which is grown commercially on lignocellulosic materials at a scale of several hundred thousand tons per year (see Chapter 22 of this book)

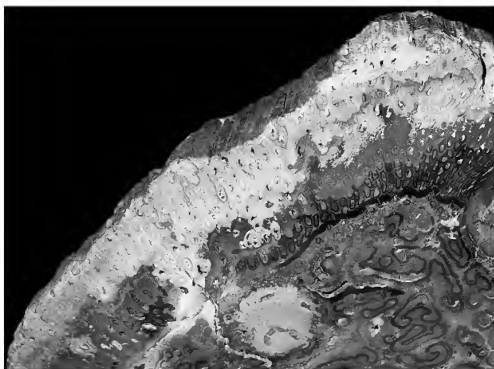


Fig. 6 Cross section of a fossil petrified palm tree from Brazil. The lignin-containing wood appears dark because of the heavy-metals which have been complexed by the lignin during the petrification. The almost pure cellulose in the periphery of the fossil trunk lacks the lignin and is free of such metals and has therefore a light colour

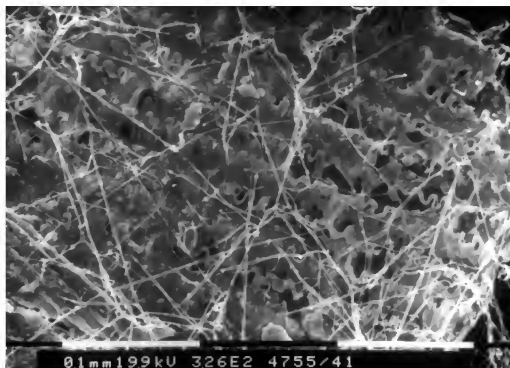


Fig. 7 Raster electron microscopy view of wheat straw after three weeks degradation by the white-rot fungus *Pleurotus ostreatus*. The filaments on the eroded straw particle are the fungal hyphae. Scale bar: 0.1 mm

almost pure cellulose and lacks the lignin fraction. Therefore, it is also free of heavy-metals and has a light colour.

Extended fungal degradation may lead to an almost complete delignification of the lignocellulosic material (Fig. 7). The attack of *Ganoderma* species on both soft and hard wood trees under the climatic conditions of Southern Andes in Chile can result finally in an almost complete delignification of the wood. There is a long tradition by the indigenous Indian population to feed their domestic animals - llamas and alpacas - with these substrates which they collected from the forests and called "palo podrido", literally translated "rotten log" (Grinbergs et al. 1979, Dill & Kraepelin 1986, Gonzales et al. 1986, Agosin et al. 1990, Schmidt 2006).

Upgrading of lignocellulose

Animal fodder is usually produced from agricultural products such as grains, cereals, by-products of the milk and sugar industries, and residues from industrial food processing. Most of the materials are further enriched with essential amino acids, vitamins, minerals, and possibly antibiotics in order to enhance animal growth (Wenk 2000, Florea & Nightingale 2004, Bouton 2007, Brufau et al. 2006, Gra-

ham et al. 2007). These additives are mainly responsible for the high costs of commercial fodder.

Raw materials for industrial fodder production such as grains and cereals represent products that are also used for human nutrition. Therefore, the industrial production of animal fodder competes in resources with direct human sustenance. Rich industrialised countries may cope with this on costs of others whereas animal production with such type of fodder in population-dense developing countries with poor nutrient supply to the people would contribute to malnutrition. In order to not touch any resources for human nutrition in animal breeding, a possible solution to the problem is the application of low quality agricultural waste of no use for human diet, thereby following the ancient principle of "palo podrido".

Low quality agricultural waste materials are poor in nutrients (proteins, vitamins) and have a high lignocellulose fibre content (Fall et al. 1998) which makes them indigestible for non-ruminants and ineffective even for feeding of ruminants. The essential step for the usage of lignocellulosic materials in animal feeding is the selective removal of lignin by white-rot fungi, thus enriching the spent material in the easily digestible cellulose or simple sugars (Gressel & Zilberstein 2003). As a further nutritional improvement of the material by the fungal treatment, protein levels and levels of essential amino acids within the fodder can increase due to the fungal biomass (Singh & Gupta 1994, Gangwar et al. 2003, Fazaeli et al. 2004a).

The idea of using fungi in improving the digestibility of lignocellulosic materials for ruminants originates from the beginning of the 20th century (Falck 1902). However, intensive studies following this idea were initiated not until the 1980th (e.g. Streeter et al. 1982, Kamra & Zadrazil 1986, Zadrazil et al. 1996) and were accompanied by better understanding of the lignocellulose degradation processes by micro-organisms.

Numerous lignocellulosic and agricultural waste materials were proven to be a potential material for fungal bio-upgrading as fodder, including the above mentioned wheat straw (Jalč et al. 1998, Fazaeli et al. 2004a), corn stalk, rice straw, cotton stalk (Kerem et al. 1992, Hadar et al. 1993), cottonseed hull (Li et al. 2001), viticulture wastes (Sánchez et al. 2002), red cedar wood (Okano et al. 2005), waste cellulose fibres (Nikolov et al. 2000), and others (see recent reviews: Jalč 2002, Villas-Bôas et al. 2002). Studied fungi included not only the edible and well studied white-rot *Pleurotus ostreatus* (Cohen et al. 2002; Fig. 5) but also other straw and wood degrading fungi, e.g. *Phanerochaete chrysosporium*, *Pholiota nameko*, *Dichomitus squalens*, *Lentinus edodes*, *Ceriporiopsis subvermispora*, *Ganoderma* spp., and *Trametes versicolor* (e.g. Zadrazil et al. 1996, Gao et al. 1997, Chantarat 2000, Flachowsky et al. 2001, Basu et al. 2002, Okano et al. 2005). Usually in such studies, improvement of *in vitro* digestibility of lignocellulosic material (IVDMD; tests in which a release of glucose from the sample by cellulases is measured) is analysed as an index to

judge the improvement of the waste material for animal feeding after fungal fermentation.

Animal feeding experiments with fungal degraded agricultural wastes are so far still scarce (Jalč 2002). Acceptance of fungal degraded material in pure form has been observed to be a problem in case of some animals. For example, pure *P. ostreatus* treated wheat straw was rejected by cattle whilst fodder mixtures with portions of up to 17% were tolerated (Adamovič et al. 1998). The level of tolerated complement in the fodder of buffalo calves is reported to be influenced by the type of treated straw - with wheat straw being preferred (Kakkar & Dhanda 1998). Partial substitutions with *P. ostreatus* treated wheat straw performed equally well or even better in nutrient intake in lactating cows compared to fodder without such complement (Fazaeli et al. 2002, 2004b). Cow's uptake of digestible organic matter from *Pleurotus* treated straw even increased when the straw was given before onset of mushroom production (Fazaeli et al. 2004a). As a drawback, *P. ostreatus* spent wheat straw was shown in steers to inhibit ammonia-N utilisation by ruminal microbes (Henics 1987). Rams fed with *P. ostreatus* var. *florida* digested wheat straw had a higher total number of bacteria in their rumens but the concentrations of cellulolytic bacteria were not changed (Montanez et al. 2004). To sheep and goat, an 100% replacement of fungi-treated rice straw had negative effects on nutrient availability (Dhanda et al. 1994, Maan et al. 1994) and, in case of *Coprinus fimetarius* (*Coprinopsis cinerea*) treated paddy straw, also on the tissue structure of animal organs (Maan et al. 2000). Also for these animals, reduction of the portions of fungal-treated straw in the diet appears to be advisable for better results of fodder turn-over (Díaz-Godínez & Sánchez 2002, Fazaeli & Masoodi 2006). Addition of organic nitrogen sources could help to further improve the digestibility of fungi-treated straw (Bisaria et al. 1997).

During fungal treatment of lignocellulosic material, cellulose and hemicellulose will also to some parts be degraded, depending on the fungal species applied. Values of cellulose losses from wheat straw have been reported as low as 6.0% (*Hericum clathroides*) and as high as 61.4% (*P. chrysosporium*). Lowest losses of hemicellulose in the range of 26-27% were observed for *H. clathroides* and *Phelinius laevigatus* and highest losses with a value of 64.7% for *P. chrysosporium*. Thus, although lignin degradation by *P. chrysosporium* is also high (62.0%), other more selective fungi leaving cellulose fairly intact are superior in production of fodder from wheat straw (Jalč 2002). Nitrogen sources tend to repress selective delignification by basidiomycetes and increase polysaccharide degradation (Reid 1989, Boyle 1998, Bisaria et al. 1997, Kachlishvili et al. 2006). Increasing the C/N ratio in the substrates by supplementation with other biomass sources possessing low nitrogen levels or elimination of nitrogen compounds from lignocellulosic material prior to fungal fermentation can therefore redirect degradation towards selective delignification. Since nitrogen is mostly bound in form of proteins that associate with the plant cells walls, one possible way to do the latter is by adding proteases to the

substrates followed by hot-water extraction of enzyme-released nitrogen. Such combined protease treatment - hot water extraction was shown to increase the selectivity of lignin decay of hemp stemwood by the white-rot fungus *Bjerkandera* sp. (Dorado et al. 2001).

As another option to enhance the digestibility of lignocellulose by ruminants, selected enzymes (e.g. laccases) might also directly be applied to lignocellulosic wastes for an *in vitro* treatment of the biomass (Ball & Jackson 1995, Niladevi et al. 2007). Furthermore, various types of enzymes (cellulases, xylanases, proteases, lipases, and others) might be used as additives to animal food ratios in order to supplement *in vivo* the rumen microbiota in degradation of lignocellulosic biomass (Krause et al. 2003, Beauchemin et al. 2004). The required enzymes, commonly enzyme mixtures, can be provided on large scale by liquid fermentations or solid state fermentations of lignocellulosic wastes by fungi. This can also include genetically optimised strains and non-edible species (see Chapter 19 of this book).

A low-cost and easy to handle process for preparation of ruminant fodder from agricultural wastes

Processing techniques for conversion of lignocellulose with fungi by solid state fermentation (SSF) have been studied and optimised at large scale technical levels (Pandey et al. 2000, Pandey 2003, Tengerdy & Szakacs 2003, Sánchez 2004; for further information see also Chapter 19 of this book). The established technologies allow commercial production of upgraded lignocellulosic materials as well as production of fungal enzymes (Robinson et al. 2001, Hölker & Lenz 2005; Chapter 19 of this book). However, present processing costs of SSF and necessary investment costs are still the most important economical barriers for production of upgraded fodder in developing countries.

A novel technique for preparation of semi-sterile lignocellulosic material for cultivation with *Pleurotus* spp. and upgrading of these materials as ruminant fodders was established in a European Union (EU)-funded project carried out by research groups from Egypt, Israel and Germany (Hüttermann et al. 2000). The goal was to develop a low cost process suitable for small farmers in the third world countries which have no high-tech equipment at hand and no access to extensive amounts of water and energy. Most important was therefore the implementation of a low-energy-cost and low-water-consuming method with cheap and simple equipment that avoids the expensive steam-sterilisation of lignocellulosic substrates prior to inoculation of the material with white-rot fungi.

The principle of the novel low-cost technique of substrate pretreatment is wetting and conditioning of lignocellulosic materials with an aqueous solution of non-toxic and food-approved detergents (e.g. Tween). This can easily be achieved by placing chopped straw or stalks into containers and rinsing the material with the detergent solution for 16-24 h (Fig. 8). Thereby, the solution is run in a closed

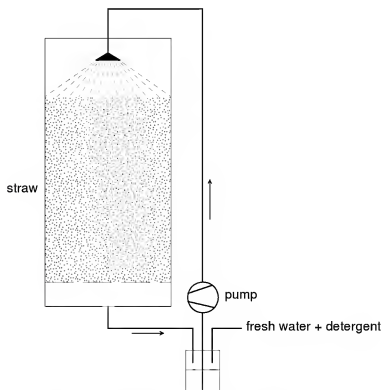


Fig. 8 Schematic drawing of the treatment of straw with a detergent solution for the preparation of fungal substrates

circuit and the water which is soaked up by the rinsed material is replaced automatically from the delivery reservoir. A small pump connected to a photovoltaic device is sufficient for processing a few hundred kilograms of material within one unit. The total investment costs for a $2 \times 2 \text{ m}^3$ test facility installed in Egypt in the years 1998- 2000 was below 250 €. Running costs are also low since energy is only required for pumping the solution at a very low speed. Furthermore, the process produces no waste water – domestic sewage can be supplied to the facility for water re-filling and the small volume of water remaining in the delivery reservoir at the end of a treatment can be used in the next incubation batch. In total, very little amounts of water are needed – only as much as required for completely wetting the substrate. Only water required for cleaning or resulting from an accident such as breakage or leakage from the container will not be utilised immediately – if collected, the water can be feed back into the regular operations.

When the rinsing process of a batch of lignocellulosic material is stopped after maximum 24 h, the lignocellulosic material is “conditioned” for an additional 24 h during which the detergent further acts destructive on microbes attaching to the wetted material and likely modifies the plant material by eliminating waxes and fats from surface hindering fungal degradation and fully wetting the material. Af-

terwards, the wet material in the container is ready for inoculation with e.g. millet infested by *Pleurotus* spp.. Upon 14 days incubation, the substrate is typically completely overgrown by the fungus (Fig. 9). Depending on the type of substrate, already after three weeks of incubation with the fungus, the material is converted into a fodder applicable to feeding of ruminants (Fig. 10).

The presented process was developed for the preparation of lignocellulosic material for incubation and upgrading with fungi at a commercial scale particularly under the conditions of a remote farm in Africa (Hüttermann et al. 2000). For the farmers which implement this process with *Pleurotus* spp. as the material processing fungus, there are two possibilities of producing a marketable product:

- High yields of fodder are gained after incubation times with the fungus as short as three weeks.
- Longer incubations lowers the yields of fodder but after about 80 days edible fruiting bodies of *Pleurotus* (Fig. 5) are obtained which are highly valued products on the local food markets and, upon mushroom harvesting, the residual substrate can still be used as fodder.

The practicability of the developed method was successfully tested under conditions of small farms in Egypt. Controlled feeding experiments with Rahmani rams (Fig. 10) and fodder from three weeks fungal incubation of rice straw were

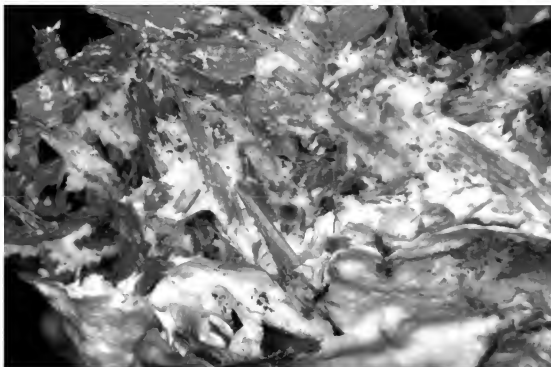


Fig. 9 Corn stalk which is colonised by mycelium of the *Pleurotus ostreatus*



Fig. 10 Rahmani ram in a metabolic cage for the testing of the nutritive value of up-graded fodder from lignocellulosic material. The animals were given mixtures of untreated and fungal-treated rice straw and allowed to eat as much as they want

accompanied by chemical analysis of the material confirming the increase of digestibility and protein content. Sheep fed with the rice straw treated with *Pleurotus* spp. gained weight even in a short time of 14 days (Table 1). The usual commercial feeding pellets were reduced by 50% and substituted with the straw, offered *ad libitum*.

Other types of agricultural wastes such as wheat straw and corn stalk can also be applied to the procedure. Even cotton straw which contains more lignin than spruce wood is transferred by the process into valuable fodder (Kerem et al. 1992, Hadar et al. 1993). In Northern Israel, Holstein dairy cows are now fed by fungal treated lignocellulose. 50% of the conventional feeding pellets can be saved by supplementing the diet of the cows with this substrate without any significant loss of milk production (Prof. Y. Hadar, Hebrew University of Jerusalem, personal communication). The newly developed low-tech process for growing fungi enables the conversion of straw and also wood chips to meat and milk even in regions with no electricity „on village, on farm“. The Egyptian Ministry of Agriculture has made up the following calculation: if the whole straw which at

Table 1 Results of a feeding experiment of Rahmani rams (three animals per treatment) with rice straw treated with white-rot fungi (adapted from Hüttermann et al. 2000)

Treatment	Weight [kg]		
	Initial	Final	Difference
Untreated straw	46.90	46.50	- 0.40
Straw treated with <i>Pleurotus ostreatus</i>	46.60	47.30	+ 0.70
Straw treated with <i>Pleurotus columbinus</i>	47.00	47.20	+ 0.20

present is produced in the Nile delta - 10 million tons - is converted to fodder for ruminants by the white-rot fungi, the Egyptian meat production could be doubled, without taking even one additional ha of land under the plough. About 150 000 new jobs would be created in the rural areas and the gain in the GNP (gross national product) of Egypt would be about 3 billion US \$ (Dr. A. Hamza, Central Laboratory for Food and Feed, Cairo, personal communication).

Conclusions

There is no dissent in the relevant literature about the fact that the grazing of semi-arid lands by small ruminants, especially goats, has a disastrous effect on the ecology of these lands (e.g. Kollmannsperger 1957, 1978, Leisinger & Schmitt 1992, Brandt & Thornes 1996, Conacher & Sala 1998, Bharara 1999). As outlined in Chapters 4 to 6 of this book, it is possible to reverse this vicious cycle of land degradation by afforestation. A main problem with such ventures is the fact that these afforestations will be not accepted by the herdsmen which use the land for feeding their cattle. Compared to the degraded vegetation which serves usually as a pasture for sheep or goats, fast growing trees produce on the same stands five to ten times more biomass. This means that after a conversion of part of the produced timber into fodder for small ruminants, the same amount of goats can be supported on the former grazing range without posing any danger to the forests. Large scale implementation of the techniques described in this chapter is thus the only way which is available at present to reconcile the needs of the herdsmen with the ecological necessity of restoration of the heavily degraded lands in Africa, Asia, and Latin-America.

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22. Mushroom Production

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Introduction

Since the stone age, mushrooms served humans as food, medicine and psycho-active drugs, religious symbols, and helpful tools. As a nice documentation, in the bag of the iceman Ötzi, a mummy discovered in 1991 in the Alps, remains of *Fomes fomentarius* (tinder polypore) and *Piptoporus betulinus* (birch bracket) were found and fragments of *Daedaleopsis tricolor* (a polypore bracket fungus) in huts of a Neolithic village north of Roma (Peintner et al. 1998, Roussel et al. 2002, Bernicchia et al. 2006). Within native traditional populations all over the world, there is tremendous inherited knowledge on mushrooms and their potential usages (Allen & Merlin 1992, Buyck & Nzigidaheha 1995, Blanchette 1997, Mahapatra & Panda 2002, Michelot & Melendez-Howell 2003, Montoya et al. 2003, Chang & Lee 2004, Zent et al. 2004, Pieroni et al. 2005, Garibay-Orijel et al. 2006, Lampman

2007) that, however, is in danger to be lost. Mushroom consumption and medical application of fungi is deep-rooted in Asian countries, also in their modern societies. In many European cultures, in contrast, mushrooms have been broadly neglected for centuries. Often, there is no practice of mushroom hunting by historical elimination of knowledge, fear of mushroom poisoning, and other reasons (Benjamin 1995, de Román et al. 2006).

Traditionally, mushrooms are collected from the wild, unless a cultivation method has been established for a species. Mushroom cultivation started in China 600 years AD with artificial inoculation of twigs with *Auricularia auricula-judae* (jew's ear fungus, wood-ear mushroom), a mushroom common to the Asian kitchen. Also *Lentinula edodes* (shiitake) was first cultivated on logs in China, around 1000 to 1100 AD (Chang 1993, Luo 2004). In Europe, mushroom cultivation came up around 300 years ago in Paris during the time of Louis XIV, when gardeners first grew *Agaricus bisporus* (button mushroom) on beds fertilised with dung and, later on, in cellars and catacombs underneath the town (Ainsworth 1976). Until now, the 'Champignon de Paris' is the most cultivated mushroom in Europe and the world (Chang 1999, Kues & Liu 2000; Fig. 1, Table 1). *Pleurotus* ssp.

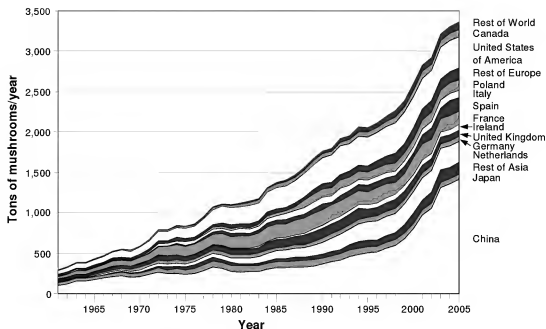


Fig. 1 Worldwide mushroom production (including interalia: *Agaricus* and *Boletus*, both basidiomycetes, and *Morchella* and *Tuber*, both ascomycetes) according to FAOSTAT data (2005). *Boletus*, *Morchella* and *Tuber* are ectomycorrhizal species that are collected from forests and plantations (Yun & Hall 2004). *Agaricus* will therefore present the majority of mushrooms in these values. This assumption is supported by the data presented in Tables 1 and 2

Table 1 Estimated worldwide mushroom production of the most important species in tons/year (Chang 1999, Kües & Liu 2000)

	1981	1986	1990	1994	1997
<i>Agaricus bisporus</i>	900.0	1,227.0	1,420.0	1,846.0	1,955.9
<i>Lentinula edodes</i>	180.0	314.0	393.0	826.2	1,564.4
<i>Pleurotus</i> spp.	35.0	169.0	900.0	797.4	875.6
<i>Auricularia</i> spp.	10.0	119.0	400.0	420.1	485.3
<i>Volvariella volvacea</i>	54.0	178.0	207.0	298.8	180.8
<i>Flammulina velutipes</i>	60.0	100.0	143.0	229.8	284.7

(oyster mushrooms, hiratake) are currently the second most cultivated group of mushrooms (Chang 2005, Tan et al. 2005, Yamanaka 2005; Table 1). *Pleurotus ostreatus* was first artificially cultured at the beginning of the 20th century by Richard Falck (1917), who in 1910 became the first forest botany professor of the “Institut für Technische Mykologie” at the Forest Faculty in Hannoversch-Münden (Hütermann 1987, 1991), the predecessor of the Section Molecular Wood Biotechnology at the Faculty of Forest Sciences and Forest Ecology in Göttingen. Cultivation of all other mushrooms domesticated up to 1900 (edible species: *Flammulina velutipes* - enoki mushroom, enokitake, velvet foot, *Volvariella volvacea* - paddy straw fungus, and *Tremella fuciformis* - ba mu erh, trembling fungus; medicinal mushrooms: *Poria cocos* - fu ling and *Ganoderma* spp. - reishi) happened first in China (Chang 1993). Since the nineteen-eighties, mushroom production booms with more and more countries being engaged in large scale mushroom cultivation (Fig. 1) and with continually increasing numbers of new species (sometimes referred to as “speciality mushrooms”) with well-established cultivation techniques (Chang 1999, Kües & Liu 2000, Chen 2004, Royse et al. 2005, Yamanaka 2005; Tables 1 and 2). Nowadays, a large variety of mushrooms are cultivated all over the world. China still occupies the leading position in mushroom production and consumption, with more than 950 different known edible species in the wild of which about 50 are commercially cultivated (Chen 2004, Chang 2005, Yamanaka 2005; Table 2). Commercial mushroom cultivation often bases on recycling of vast amounts of agro-forestry wastes. The variety of wastes reach from straw (wheat, rice, oat, ...), reed grass, sawdust of different species (spruce, pine, beech, birch, pine, gum wood, ...), banana and bamboo leaves, tree bark and stems, several husk types, some scrubs, and others (reviewed by Poppe 2000). Cultivation of speciality mushrooms on lignocellulosic wastes represents one of the most economically and cost-effective organic recycling processes (Poppe 2000, Philippoussis et al. 2001, Mandeel et al. 2005; see also Chapter 20 of this book).

Commonly, mushrooms are understood as fruiting bodies of fungi that are edible (mushrooms) or poisonous (toadstools). Chang and Miles (1989) defined a mushroom being a macrofungus with an epigeous (above ground) or hypogeous

Table 2 The twelve mostly produced mushrooms in China: production in tons/year (Chang 2005)

	1986	1998	2000	2001	2002	2003
<i>Pleurotus</i> spp.	100	1,020	1,700	2,590	2,647	2,488
<i>Lentinula edodes</i>	120	1,388	2,205	2,072	2,214	2,228
<i>Agaricus bisporus</i>	185	426	637	743	923	1,330
<i>Auricularia</i> spp.	80	491	968	1,124	1,242	1,655
<i>Volvariella volvacea</i>	100	32	112	116	151	197
<i>Flammulina velutipes</i>	10	189	299	389	506	558
<i>Tremella</i> spp.	50	100	103	114	138	184
<i>Hericium erinaceus</i>	50	28	6	9.5	13	31
<i>Hypsizygus</i> spp.	-	21	84	120	190	243
<i>Pholiota nameko</i>	1	31	48	51	85	172
<i>Grifola frondosa</i>	-	10	6	15	37	25
<i>Coprinus comatus</i>	-	-	-	39	157	178
Others*	-	664	470	371	464	1,100

*Others include *Pleurotus nebrodensis*, *Pleurotus eryngii*, *Agrocybe chaxinggu*, *Dictyophora* spp., *Agaricus brasiliensis*, *Ganoderma* spp., *Wolfiporia cocos*, *Lepista nuda*, *Agrocybe aegerita*, *Tricholoma giganteum*, *Auricularia fuscossuccinea*, *Tremella cinnabarina*, *Pleurotus citrinopileatus*, *Pleurotus sapidus*, *Stropharia rugoso-annulata* and *Lentinus giganteus*

(below ground) fruiting body, which can be seen by naked eye and picked by hand. In nature, more than 12,000 fungal species can be declared as being mushrooms, of which around 2,000 to 2,500 are estimated to be edible with a more or less savoury taste. Most of the mushrooms belong to the basidiomycetes whilst a few, including truffles and morels, are ascomycetes (Chang 1999, Yun & Hall 2004). All mushrooms being commercially produced on lignocellulosic wastes are basidiomycetes. In contrast, the black truffle, *Tuber melanosporum*, is an ascomycete that cannot be cultivated in free culture but is fostered as an ectomycorrhizal species in combination with hazelnuts and oak trees. For further development, *in vitro* mycorrhized trees should be planted into fields with calcareous soils with particular properties. After 4 to 6 years, the first ascocarps (fruiting bodies of the ascomycetes) occur, but harvests start not before 10 years after plantation (Shaw et al. 1996, Olivier 2000, Yun & Hall 2004, Bonet et al. 2006, Mello et al. 2006). Nowadays, more than half of the black truffle come from commercial plantations (Hall et al. 2003). *Tuber formosanum* is a related species endemic to Taiwan that is ectomycorrhizal to the Fagaceae *Cyclobalanopsis glauca* and cultivated in man-made trufferies (Hu et al. 2005). In a few instances, fruiting bodies of ectomycorrhizal basidiomycetes have been obtained in pot-culture with a respective host (Danell & Comacho 1997, Yamada et al. 2001; see below for further information).

Because mushrooms have a high content of crude fibre, crude protein (usually 20-30% of dry matter), B vitamins, low fat, and nearly no cholesterol (for example

see Braaksma & Schaap 1996, Manzi et al. 1999, Chiu 2000, Mattila et al. 2001, Sanmee et al. 2003, Adejumo & Awosanya 2005, del Toro et al. 2006, de Román et al. 2006, Nwanze et al. 2006), they present a highly valuable food. Besides, mushrooms serve as medicine and for prophylaxis to keep good health. Fruiting bodies as well as fungal mycelia contain several bioactive compounds. For instance, fungal polysaccharides are recognised to have anti-tumor effects by activating various immunoresponses. Most of these bioactive polysaccharides belong to the β -glucans, like Lentinan from *L. edodes* and Schizophyllan from *Schizophyllum commune* (split gill fungus), both of which are commercially available (Wasser 2002, 2004). Chang and Buswell (1996) coined the word “nutraceuticals” to indicate the dual roles of edible mushrooms being natural food and facilitators of maintaining good health. On the other hand, the term “nutriceuticals” is used for mushroom or mycelium extracts that possess both nutritional and medicinal attributes and, being incorporated into a capsule or tablet, are consumed as a dietary supplement for therapeutic purposes.

General biology of mushrooms

Breeding and life cycle

Genetic breeding of mushrooms targets at optimisation of growth and fruiting body yields, at nutritional and structural quality, texture and taste of mushrooms, disease resistances, and shelf lives. It makes use of the gene pool of a species by sexual reproduction and thereby genetic recombination (Kitamoto 2006). The breeding systems and, connected with them, the life cycles of mushrooms are very diverse (Fig. 2) and have to be studied in detail for each new fungus.

Principally, the life cycles of basidiomycetes are controlled by either of two different breeding systems: **homothallism** and **heterothallism** (Fig. 2). Species are **inbreeding** (sexual reproduction within very closely related or identical individuals that serves genome conservation) when fruiting bodies develop on a mycelium that germinated from a single sexual spore (meiotic spore), in basidiomycetes the basidiospore. In case the basidiospore contained one **haploid** nucleus (a **uni-nucleate** spore that contains one nucleus with one set of chromosomes) or two identical haploid nuclei (a **binucleate** spore), the resulting mycelium is a **homokaryon** (mycelium with one type of nuclei) that is **self-fertile**. This breeding system is called **primary homothallism**. **Secondary homothallism** (or **pseudo-homothallism**) simulates self-fertility of a mycelium. It occurs in a situation where two types of haploid nuclei of different **mating types** (physiological properties that determine sexual compatibility - only unlike mating types are sexually compatible) are present in binucleate basidiospores. The germinated mycelium from such a spore is therefore a **fertile dikaryon** that is characterised by two distinct haploid nuclei in each hyphal cell and the ability to produce fruiting bodies with sexual basidiospores. The majority of the fungi is, however, heterothallic and

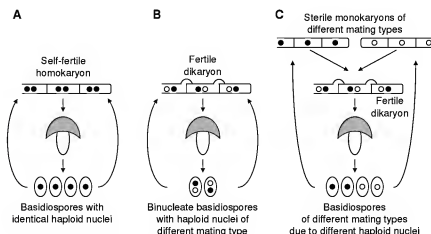


Fig. 2 Schematic representation of main types of life cycles in the basidiomycetes (Whitehouse 1949, Kues 2000, Kues & Liu 2000, Walsers et al. 2000). Generally, nuclear fusion (karyogamy) of two haploid nuclei (with one set of chromosomes each) occurs in specialised cells (basidia) within the fruiting bodies which results in a single diploid nucleus (with two sets of chromosomes). Directly after, the diploid nucleus undergoes the two divisions of meiosis which reduces the nuclear status back to haploid. After meiosis, four haploid nuclei are present in the basidium that migrate into the four basidiospores formed on a basidium (see also Fig. 3). In many species as an extra complication, a post-meiotic mitosis duplicates the number of haploid nuclei either in the basidium before nuclear migration or in the basidiospores after the migration of nuclei. As a consequence, each single basidiospore can contain two haploid nuclei (see Fig. 3). For simplification in this figure, only the four direct products of meiosis are shown. Nuclei within cells are shown by filled and open circles. The same colouration indicates genetic identity with respect to mating types. A. A self-fertile, i.e. homothallic homokaryon produces basidiospores with genetic identical haploid nuclei. These germinate into a self-fertile mycelium able to form fruiting bodies with basidia in which identical haploid nuclei fuse before meiosis. B. A secondary homothallic species typically has only two basidiospores per basidium into which two different haploid nuclei of different mating type migrate. The fertile dikaryon that germinates from such a basidiospore usually has clamp cells. Clamp cells are special structures formed at the hyphal septa to ensure that each hyphal segment in the dikaryon receives one haploid nucleus of each mating type (Iwasa et al. 1997, Badalyan et al. 2004). C. Heterothallic basidiomycetes produce basidiospores of different mating types that germinate into sterile monokaryons. Only when monokaryons of different mating types fuse, a fertile dikaryon with clamp cells is formed on which fruiting bodies may develop. If the mating type is determined by one genetic locus (bipolar species), only spores with paternal mating types arise from the mushroom. If two independent loci control mating types (tetrapolar species), four types of spores are observed, two with the parental mating types and two that have new mating types. Either monokaryons of the parental mating types or monokaryons of the new mating types can fuse (Raper 1966, Kues 2000, Kothe 2001; Chapter 23 of this book)

outbreeding (sexual reproduction between not closely related individuals which serves mixing of the gene pool). The mycelia germinated from basidiospores of heterothallic basidiomycetes are **infertile monokaryons** (specific homokaryons with only one haploid nucleus per hyphal cell). In such species, two sterile monokaryons of different, compatible mating types need to fuse in order to form a fertile dikaryotic mycelium able to produce fruiting bodies (Whitehouse 1949, Kűes 2000, Kűes & Liu 2000, Walser et al. 2000). In the fruiting body by karyogamy and meiosis, the genes of the genomes of the two parental monokaryons will be mixed and transferred in various new combinations into the basidiospores. It is therefore relatively easy to breed new lines of mushrooms with new properties (e.g. higher yields of fruiting bodies, better shape and size of fruiting bodies, etc.) from heterothallic species that fruit in culture, unlike from homothallic species (Kűes & Liu 2000, Kothe 2001).

As an example for the different stages in a life cycle of a heterothallic basidiomycete, the best understood species *Coprinopsis cinerea* (formerly *Coprinus cinereus*, dunghill ink cap mushroom) is given in Fig. 3. Since it is outbreeding, this species serves researchers as a model fungus to unravel the physiological, cytological and genetic background of mushroom development (Kűes 2000; see Chapter 23 of this book) but it is also edible. It is cultivated on agricultural wastes as Hed Cone Noy (little stubble mushroom) in the humid seasons in Thailand by small family business (see Chapter 23 of this book) and it is eaten by workers on sisal plantations in Africa where the fungus fruits naturally in high numbers on compost heaps of the Agave plantation wastes (Härkönen et al. 1993). Consumption is also been recorded from China (Hall et al. 1998a). Rice fermented with *C. cinerea* is said to have hepatoprotective activity against chemicals (Lee et al. 2004). Furthermore, under the name *Coprinus fimetarius*, the fungus was used in the Karmal process in India to ferment straw for animal fodder (Maan et al. 1994; see Chapter 21 of this book). Since not (yet) of major economical interest, genetic breeding in this species has in the past mainly been done for research purposes (see Chapter 23 of this book). From this work it is however clear that there are large variations in the worldwide gene pool of the species. For breeders it is important to note that many poorly performing genes in growth and development reside within the natural gene pool which makes breeding of a new line with specific properties a long-lasting and laborious process. Such genes of “bad quality” are maintained hidden in nature and are obviously not easily lost from populations but transferred into new progenies. This is, because a dikaryon with two different haploid nuclei in its cells is reacting like a diploid organism with genetic complementation between the two sets of parental chromosomes (North 1970, Moore 1980, Liu et al. 1999, Muraguchi et al. 2001, Srivilai 2006). Thus, it is enough if only one of the nuclei carries a functional allele (allele = one specific form of a given gene).

Typically, fungal breeding is carried out by an empirical trial and error process. Breeding of a new hybrid strain from any heterothallic basidiomycete is principally

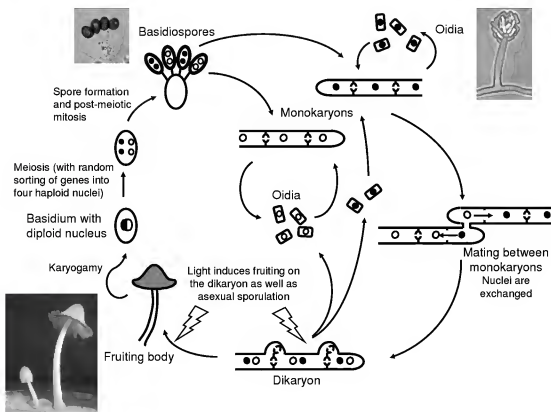


Fig. 3 Life cycle of *Coprinopsis cinerea* (after Kues 2000). The life cycle starts with germination of basidiospores into sterile monokaryons with one type of haploid nuclei in the hyphal cells. By fusion of two mating-compatible monokaryons with nuclei of different mating type (indicated by filled and open circles), a dikaryon arises that carries in the hyphal segments each one parental haploid nucleus. On the dikaryon, the fruiting bodies (see photo, lower corner left) develop under defined environmental conditions (nutrient depletion, 25–28 °C, day–night rhythm, >80% humidity). Karyogamy and meiosis occur in the basidia, the four haploid nuclei migrate into the basidiospores and undergo a post-meiotic mitosis. The basidiospores stain black by melanin incorporation in the cell walls (see photo, upper left corner) and this also gives the mushroom cap its colour. In the sexual cycle of reproduction, the two parental genomes are mixed and transferred in various new gene combinations to the progeny. Due to the two independent mating type loci (called A and B), basidiospores with four different mating types can arise from a single fruiting body (further explanations in Chapter 23 of this book). Unlike many other species, *C. cinerea* in addition has subsidiary asexual cycles of reproduction. Asexual, mitotic spores called oidia are constitutively produced in high numbers on the monokaryon on specialised aerial structures (oidiophores, see upper right corner for a photograph) and, upon light induction, in small numbers also on the dikaryon. The nuclei in oidia and in the monokaryons germinated from oidia are genetically identical to those present in the mycelium the spores arrived from. Oidia serve in nature in the distribution of the fungus by flies

performed by four successive processes: i. the selection of two parental dikaryons (the prevailing mycelium in nature), ii. the production of monokaryotic line stocks from those parental dikaryons, iii. the production of various hybrid dikaryons by crossing between two selected monokaryotic line stocks, and iv. several levels of cultivation tests of the crossing products for selecting a superior hybrid strain (Kitamoto 2006). Such breeding programmes for example revealed in the outbreeding tetrapolar species *P. ostreatus* the occurrence of many quantitative trait loci (QTLs) whose alleles (different forms of a gene) determine variability in growth speed and/or mushroom yields. Molecular RAPD markers (random amplified polymorphic DNA markers; see Chapter 8 of this book for detailed explanation) were defined in order to easily follow up such genetic traits in progenies of this species (Larrraya et al. 2002, 2003, Sonnenberg et al. 2005, Park et al. 2006). Similar breeding programmes with development of molecular markers exist also for other outbreeding mushrooms. Further to breeding, RAPD and other molecular markers can be used to define and recognise individual commercial strains within species (Zang & Molina 1995, Terashima et al. 2002, Yan et al. 2003, 2004, Yan & Jiang 2005, Qin et al. 2006). Breeding programmes of heterothallic species target at various properties such as at better substrate colonisation, faster growth and higher production rates (Larrraya et al. 2001, 2003, Park et al. 2006), better thermotolerance (Guler et al. 2006), cap colour (Moquet et al. 1997), loss of spore production (Callac 1995, Baars et al. 2004, Sonnenberg et al. 2005), better antioxidative properties (Kong et al. 2004), and resistance against pests (Tokimoto & Komatsu 1995, 1998, Anderson et al. 2001).

Molecular markers might also be defined for identification of individual strains of homothallic (e.g. *V. volucaeae*, Chiu et al. 1995) and secondarily homothallic species (e.g. *A. bisporus*; Calvo-Bado et al. 2001, Moore et al. 2001, Ramírez et al. 2001). Moreover, the same breeding procedure as described above for the heterothallic species is principally possible with secondarily homothallic species but it requires much more effort. Next to the preponderant two-spored basidia, such species to a low degree do form aberrant three- and four-spored basidia with some of the basidiospores carrying only one type of haploid nuclei. Upon germination, such irregular spores will form a monokaryon. By a reduced growth speed, these rare spore events might easily be identified within germinated progeny and used for crosses. However, the ability to mate with other isolates and the frequency of recombination is often reduced in secondary homothallic species as found for many isolates of the button mushroom *A. bisporus* var. *bisporus*. This further can hamper breeding programmes (Challen & Elliott, 1989, Kerrigan et al. 1992, 1993, 1994, Stoop & Mooibroek 1999, Kües & Liu 2000, Calvo-Bardo et al. 2001, Callac et al. 2006, Mazheika et al. 2006). In consequence, few commercial strains are available for *A. bisporus* (Stoop & Mooibroek 1999, Anderson et al. 2001). Mushroom production to a large degree is done with the white hybrid strains Horst U1 and U3, isolated with much patience by Fritsche (1983). With the detec-

tion of a four-spored variety of the button mushroom in the wild (*A. bisporus* var. *burnettii*), the situation for breeding has now much been eased (Sonnenberg et al. 2005).

Growth types of fungi

Concerning their life-style and source of nutrients, fungi can be divided into three groups: saprotrophs, symbionts and parasites.

Some of the highest priced mushrooms, e.g. *Boletus edulis* (king bolete), *Cantharellus cibarius* (chanterelle) and *Tricholoma matsutake* (matsutake) and the related *Tricholoma magnivelare* (American matsutake) belong to the basidiomycetous symbionts, whereas *T. melanosporum* (black truffle) and *Tuber magnatum* (white truffle) that can achieve prices as high as US \$ 13,000/kg; Hall et al. 1998b, 2003) belong to the mycorrhizal ascomycetes producing hypogeous fruiting bodies. Symbiotic basidiomycetes are living in a mycorrhizal association with plants and enclose their roots with mycelium. The mycelium has the capability of exchanging nutrients with the plants and protecting the roots against stress including other micro-organisms (Hampp et al. 1999, Tagu et al. 2002). Efforts have been made to cultivate mycorrhizal mushrooms without trees but with little success and promise for the future (Danell & Comacho 1997, Yamada et al. 2001, Yun & Hall 2004; see also Chapter 23 of this book). In case of matsutake, measures such as covering plots by plastic vessels, keeping the temperature around 20°C and watering the plots are applied to positively influence natural mushroom production in the forests (Kües & Liu 2000, Yun & Hall 2004). Recently, the ectomycorrhizal symbiosis has been established *in vitro* between *T. matsutake* and *Pinus densiflora*, opening up the possibility of plantation of seedlings infested with a fungus (Yamada et al. 2006) whose fruiting bodies can be priced in early season as much as US \$ 1275 per kg (Yun et al. 1997) - prices of US \$ 40-500/kg are quite common (Hall et al. 2003). The ecology of various other ectomycorrhizal species (including the king bolete and chanterelles) are studied which might help to find suitable measures in forest management for enhancing production also of these other species (Baar & ter Braak 1996, Bergemann & Largent 2000, Smit et al. 2003, Salerni & Perini 2004).

Parasitic mushroom species such as *Armillaria mellea* (honey fungus) attack living trees in order to live necrotrophic on deadened plant material (Shaw & Kile 1991). Because they damage or even kill their hosts, cultivation of such mushrooms with living trees is inapplicable. However, some less aggressive facultative pathogens will only affect weakened trees and otherwise exercise a saprotrophic life style. Species such as the edible *A. auricula-judae* and some medicinal *Ganoderma* spp. can therefore be cultured on dead wood (Oei 2003). Most of the cultivated mushrooms however use only dead organic material as a nutrient source and, thus, are fully saprotrophic organisms. They decompose complex organic matter from plants and animals by secreting enzymes that have the ability to degrade the orga-

nic matter into nutrients and minerals which the fungal hyphae for their benefit manage to take up into their cells. In the group of saprotrophic organisms, the wood-decaying fungi take a special position in mushroom production. Wood-decaying fungi are divided into white-rot and brown-rot fungi. Brown-rots lack the ability to degrade lignin. Upon decay of cellulose and hemicellulose from the wood by brown-rots, lignin is left in form of red-brown cubicles (hence the name). White-rots in contrast attack lignin as well as cellulose and hemicellulose (further reading in Chapters 17 and 19 of this book). The majority of the commercially grown mushrooms belong to the white-rots: the oyster mushrooms, shiitake, enoki, the paddy straw mushroom, and others. These wood-rotting fungi have enzymatic systems giving them the ability to grow on and degrade complex organic matter like straw, wood and bagasse. Another group of mushrooms, for example *Agaricus* species and many strains of *C. cinerea*, apparently lack the enzymatic systems that are very aggressive to lignin. These fungi therefore need less lignified and partially degraded material (straw, dung, litter) to grow on.

General aspects of mushroom cultivation

Commercial mushroom production requires fungal strains with excellent cultivation properties, robust diseases resistance, and optimum yields in a reasonable time and that provide high-quality products of appealing taste, odour, shape, texture, and good shelf life. Various strains from different fungal species are available for commercial production. The genotypic and phenotypic maintenance of the properties of the strains is highly important for a reproducible production process. In addition, isolation of new strains (germ plasms) from the wild serves breeding of new production lines with combinations of properties adapted to the special needs (Oei 2003, Singh et al. 2004, Sonnenberg et al. 2005, Yamanaka 2005, Wang 2005; see above). Furthermore, new species might be introduced to the market either from other countries (Oei 2005, Wang 2005) or by establishing cultivation conditions for new species and breeding of suitable production strains by crosses of isolates from the wild (Oei 2003, Royse et al. 2005, Yamanaka 2005). Preferences of consumers in tastes will influence the choice of mushrooms for cultivation. Appropriate marketing serves promoting new species (Oei 2003, 2005, Royse et al. 2005).

Next to suitable fungal isolates, most important factors to be considered in successful and economical mushroom cultivation (Fig. 4) are the substrate and the environmental conditions (temperature, humidity, aeration, pH of the substrate) needed for mycelial growth and fruiting (see below). On-hand resources are affecting the choice of substrate. For example, wood from local fast growing trees provides substrate in Hawaii for cultivation of *P. ostreatus*, whereas various types of straw (e.g. wheat, rice, maize, banana leaves, pumpkin, etc.) as wastes from local agricultural practices are used for cultivation of the fungus in Europe, Asia, Latin

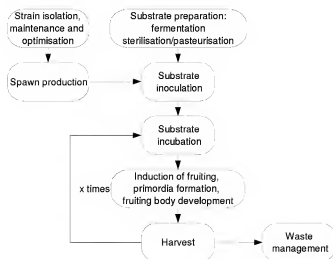


Fig. 4 General scheme of the main operational steps in mushroom production (modified from Sánchez 2004)

America or Africa (Zadrazil 1997, Obodai et al. 2003, Bonatti et al. 2004, Mendez et al. 2005, Salmones et al. 2005, Pant et al. 2006, Tisdale et al. 2006). Possibilities of investment play another major role in how to cultivate mushrooms. For example, seasonal cultivation of mushrooms should be considered if high installation costs for specific cultivation chambers are not affordable. *Agaricus bitorquis*, *C. cinerea*, *Pleurotus abalonus* and *V. volvacea* with temperature requirements for fruiting at 25°C and above are ideal species for cultivation in tropical countries during humid seasons (Chang & Quinio 1982, Oei 2003; W. Chaisaena, personal communication). In temperate climates, heating costs can be avoided by cultivating species favouring lower temperatures for growth and fruiting. Therefore, existing structures can be used, such as natural caves, defence tunnels, bunkers (see Fig. 5) or other isolated buildings that reduce heating costs in winter, respectively cooling costs in summer (Oei 2003). Furthermore for a year-round-production, cold-tolerant and thermo-tolerant strains of a species might be used in turn in winter and in summer, as done in China with different *L. edodes* strains (Li 2005).

Mushroom spawn

The success of commercial mushroom production is not only influenced by the fungal strains and the conditions under which they produce mushrooms but very much also by the type and quality of the cultures taken for inoculation (Fig. 4). Mycelial cultures on grain (e.g. rye, wheat or millet), wood sticks, or liquid substrates are variably used as the inoculum known as spawn, i.e. the starting material to rapidly colonise the growth substrate with a fungus (Royse 1985, Shieh et al. 1991, Rosado et al. 2002, Sánchez 2004, Nwanze et al. 2005, Sainos et al. 2005). Although a large cost factor, mushroom growers usually buy the spawn from





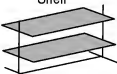
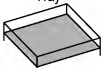
Fig. 5 A former army bunker offers ideal growth facilities for commercial production of *Pleurotus ostreatus*. Left: the entrance of one of the bunkers used by 'druid Austernpilze' (Immichenhain, Germany); right: view into the bunker with several hundreds of straw bags on which mushrooms are forming (second flush)

special companies concentrating on strain improvement and spawn production, since the knowledge and equipment needed for maintaining production strains and producing spawn is complex and time-consuming. Spawn needs to be produced under sterile conditions to diminish contamination of the substrate (Sánchez 2004). Only when commercially offered spawns lack a required quality or when special mushrooms are cultivated, it is recommendable for the mushroom growers to produce also their own spawn (Oei 2003).

Substrate

Unless mushrooms are grown on wood logs, the preparation of the growth substrate is one of the most important issues in mushroom cultivation. The individual physiological capabilities of the species to attack lignocellulosic waste materials decide upon the choice of substrates (Table 3). A comprehensive list of possible substrates with more than 200 agro-forestry wastes for the usage in mushroom cultivation is given by Poppe (2000). Depending on the character of the waste material, the biological efficiency - i.e., the total weight of obtained fresh mushroom compared to the total weight of dried substrate - might differ (Table 4). Lignocellulosic wastes are usually low in nitrogen. Cereal straw for example has a nitrogen content of only 0.59-0.66% (Stamets 1993). Mushrooms usually perform better on those of the wastes that have a higher nitrogen content (Tisdale et al. 2006). Addition of nitrogen-rich supplements (inorganic and organic) such as ammonium sulphate, ammonium nitrate, urea, or organic nitrogen sources such as fish meal (10.2% N), soy bean meal (>7% N), chicken manure, and brewer grain (3.2-4.4% N) to improve the C/N rate in a growth substrate can therefore positively influence mushroom yields (Stamets 1993, Noble & Gaze 1996, Rajarathnam et al. 2001, Noble et al. 2002, Demirer et al. 2005, Tan et al. 2005). Growth substrates

Table 3 Substrates for mushroom production, their handling and examples of use

Substrate	Treatment	Type of cultivation	Cultured species
Sawdust (beech, oak, ...)	Sterilisation/ autoclaving	Bag* 	<i>Pleurotus</i> spp., <i>Ganoderma</i> spp., <i>Flammulina velutipes</i> ,
Straw (wheat, rye, rice, oat, barley, cotton, ...)		Bottle* 	<i>Tremella fuciformis</i> , <i>Hericium erinaceus</i> , <i>Auricularia</i> spp.
Industrial and agricultural lignocellulose-wastes (coffee pulp, coffee waste, cotton stalks, paper, ...)	Pasteurisation	Shelf 	<i>Pleurotus</i> spp., <i>Stropharia rugoso-annulata</i> , <i>Volvariella volvacea</i>
Manure (horse and chicken dung) Soil (as compost or casing layer)	Composting/ fermenting	Tray 	<i>Agaricus</i> spp., <i>Coprinus comatus</i> , <i>Lepista nuda</i>

* Filters in bags and bottles for air sterilisation are indicated by the differential shading

might also be supplemented with micronutrient fertilisers. Manganese ions - known to stimulate the actions of certain peroxidases in lignocellulose degradation (see Chapter 17 of this book) - were shown to be the mineral of primary importance (Royse et al. 2004, Weil et al. 2006). In some instances as for *A. bisporus* and *Coprinus comatus* (shaggy mane), composting prior to use helps a better acceptance of the substrate by a fungus (Oei 2003). Lime and gypsum are added to composts in order to stabilise the pH value (Lelley 1991, Stamets 1993, Oei 2003, Mandeel et al. 2005). Of further influence is the water potential of the substrate, which determines the chance of water uptake by fungi from the substrate (Kalberer 1987, Badham 1989, Ohga 1999).

To avoid microbial contamination and other pests, sterilising of the substrates is required. The technique of sterilising, either fermentation, pasteurisation or pressure steam sterilisation (autoclaving) depends on the substrate, the fungus to be grown, the available equipment for sterilisation and also the packing system used for the substrate (Sánchez 2004, Table 3). Wheat straw for *P. ostreatus* cultivation for example is normally pasteurised or sterilised for the commercial cultivation (Stamets 1993, Oei 2003), although the fungus can be also cultivated on composted material deprived of troublesome contaminants (Philippoussis et al. 2001, Hernandez et al. 2003). After bulk pasteurisation, a substrate is normally inoculated with spawn and packed into bags or wrapped in plastic foil. Alternatively,

Table 4 Efficiencies in mushroom production of *Pleurotus* species and *Lentinula edodes* on various lignocellulosic substrates

Mushroom	Substrate	Biological efficiency [%]	Reference	
Log cultivation				
<i>Pleurotus ostreatus</i>	<i>Fagus sylvatica</i>	21.31	Pavlik 2005	
	<i>Populus tremula</i>	14.84		
	<i>Betula pubescens</i>	8.74		
	<i>Carpinus betulus</i>	8.97		
	<i>Alnus glutinosa</i>	2.60		
Bag cultivation				
<i>Pleurotus ostreatus</i>	Common reed	82.1	Gezer et al. 2005	
	Brewer grains + wheat straw	66.2	Iwase et al. 2000	
	Wheat straw	100	Fischer 2006	
	Wood chips of			
	<i>Falcataria moluccana</i>	85.6	Tsidale et al. 2006	
	<i>Casuarina equisetifolia</i>	81.5		
	<i>Eucalyptus grandis</i>	77.6		
	<i>Psidium cattleianum</i>	57.7		
	<i>Trema orientalis</i>	97.9		
	<i>Pleurotus citrinopileatus</i>	Cottonseed hulls/ wheat straw + Mn	123.5	Royse 2004
	<i>Pleurotus sajor-caju</i>	Paddy straw	109.3	Singh 2000
Wheat straw		61.3		
Grass		49.8		
<i>Lentinula edodes</i>	Sugar cane bagasse	93.8	Fung et al. 2005	
	<i>Eucalyptus</i> sawdust	76.0		
	Oak sawdust	66.0		

a substrate is first packed, then sterilised and afterwards inoculated with an appropriate type of spawn (Oei 2003, Fig. 6). The amount of spawn needed for inoculation depends on the species, the experiences of the growers and the quality of the spawn. Particularly for the abundantly produced species, substrate preparation and inoculation is nowadays often outsourced from the mushroom farms (Fig. 6; K.-D. Hesse, personal communication).

Growth and production conditions

After inoculation of the substrate, an incubation phase follows that gives the mycelium the opportunity to completely colonise the substrate (Fig. 4). This primary incubation phase is normally the longest lasting step, since the mushroom has to adapt its metabolism to the new environment and to spread over the substrate (Philippoussis et al. 2003). The individual parameters (temperature, relative air hu-

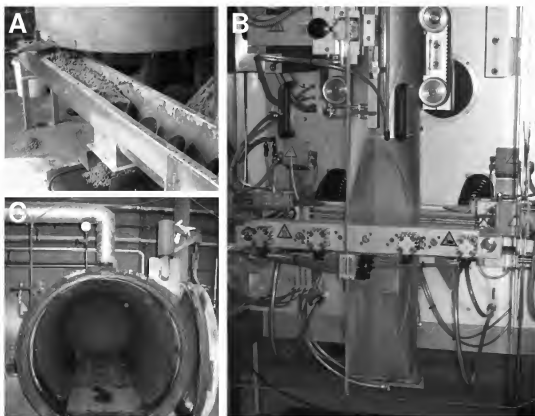


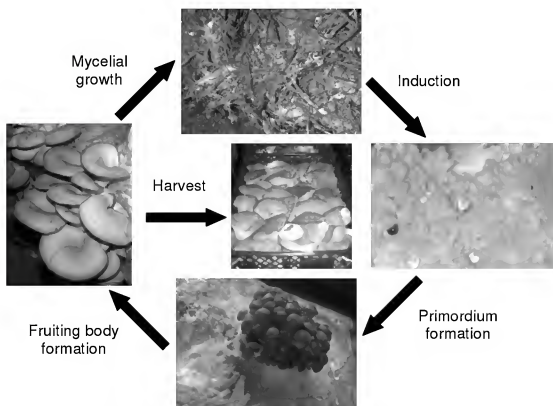
Fig. 6 Machinery for substrate preparation at the company 'Hesse Pilzsubstrate' (Schweinsberg, Germany). A. Mixing of sawdust with required supplements and delivering the ready substrate to the filling machine (B.) where it is filled into propylene bags. C. Autoclave used for steam-sterilising the packed substrate

midity, length of incubation) for the incubation phase depend on the fungal species cultivated (Table 5). However, within a given species the optimum growth conditions can also vary due to different genetic backgrounds of strains. African isolates of *A. bisporus* for example grow best at 30°C whereas isolates from temperate regions prefer 23 to 25°C (Date & Mizuno 1997, Guler et al. 2006).

Following the incubation phase for mycelial growth, formation of primordia (compact aggregated structures that present early developmental stages in fruiting body production; see Fig. 7) is induced by lowering the temperature and guaranteeing a high humidity on the surface of the substrate (Table 5). Also, a decline of the CO₂ level in the air can be helpful for mushroom production of *A. bisporus* and *P. ostreatus*. In case of *L. edodes*, a shock by drowning the substrate for 24 h in water supports primordia induction (Chang & Hayes 1978, Stamets 1993, Oei 2003). Light is another very important control factor in mushroom production. Whilst mycelial growth usually occurs in the dark, formation of primordia depends

Table 5 Cultivation conditions of some selected mushrooms (after Lelley 1991, Stamets 1993)

	<i>Volvariella volvaceae</i>	<i>Pleurotus eryngii</i>	<i>Pleurotus ostreatus</i>	<i>Lentinula edodes</i>	<i>Agrocybe aegerita</i>
Mycelial growth					
Temperature [°C]	24-35	25-28	24	21-27	21-27
Relative air humidity [%]	80-95	55-95	85-95	95-100	95-100
Duration [days]	5-10	28-56	12-21	35-70	20-28
Primordia formation					
Temperature [°C]	27-32	10-17	10-16	10-16	10-16
Relative air humidity [%]	90-100	90-100	95-100	95-100	95-100
Duration [days]	4-6	4-5	3-5	5-7	7-14
Fruiting body maturation					
Temperature [°C]	27-32	12-26	10-21	16-18	13-18
Relative air humidity [%]	85-95	80-90	85-90	60-80	90-95
Duration [days]	6-10	21-35	4-7	5-8	4-6
Harvest					
Flushes	>2	2	3-4	4	2

Fig. 7 The main steps in mushroom cultivation documented for *Pleurotus ostreatus* on pasteurised wheat straw

on low levels of light. Too much light in contrast can hinder mushroom production (Kitamoto et al. 1993).

For maturation of the fruiting bodies (Fig. 7), for some of the species, the cultivation conditions have once more to be changed (Table 5). Slightly increased temperatures help mushrooms to develop nicely and lowering of the relative humidity helps to avoid unsightly spots on the caps and bacterial and fungal infections. However, in specific cases such as *F. velutipes* and *C. cinerea* where fully developed mushrooms are unwanted by their poor shelf-life, light is withdrawn with the effect that long-lasting elongated stipes with underdeveloped caps are formed that can be better marketed (Oei 2003; see Chapter 23 of this book).

After harvesting, further rounds of mushroom production can take place on the same batch of substrate, initiated by new growth incubation phases followed by phases of primordia induction and formation of fruiting bodies (Fig. 4 and Fig. 7, Table 5). With increasing numbers of production periods (known as flushes), yield and quality of mushrooms can decrease so that commercial exploitation after the second or third flush and sometimes even after the first flush is not of economical benefit [Ohga & Royse 2004; druid Austernpilze (Immichenhain, Germany) and Lehr Speisepilzkulturen (Schwalmtal, Germany), personal communications]. After the last harvest, the spent mushroom substrate (SMS) has to be disposed (Fig. 4).

Recycling of spent mushroom substrate (SMS)

As in other industrial production processes, waste management is also an important environmental and economical factor in mushroom cultivation. SMS might be disposed by transportation onto agricultural land. On the fields, it can act as a soil conditioner and, upon further degradation, it can have fertiliser effects on growth of the crops (Stewart et al. 1998a,b, 2000). However, the possibility of negative ecological effects by application of SMS such as alteration of the soil microbial community (Watabe et al. 2004) and contamination of groundwater by leachates of nitrates and others solutes have to be considered (Guo 2005).

After mushroom cultivation, SMS has still a considerable economical value. In regions with a high density of mushroom farming, like in the southeast of Pennsylvania in the USA, a market for SMS as a soil supplement for various crops and as a potting medium for seedlings and ornamental shrubs in containers has been established. Mostly, spent compost of *A. bisporus* cultivation is used. Pasteurised before removing it from the mushrooms growth rooms, it gives a half-sterile, nutrient-rich plant growth substrate (Chong 2005; see Chapter 24 of this book).

Enriched with cotton seed meal, soya meal or another suitable cheap nitrogen source and by changing the fungal species (e.g. from *Pleurotus* to *Stropharia*, *Agaricus* to *Volvariella*, *Volvariella* to *P. sajor-caju*, *F. velutipes* to *C. comatus*, etc.), SMS might be subjected to mushroom re-cultivation (Sánchez 2004). Straw-based SMS can

further serve as animal feed (Fazacli et al. 2004, Fazacli & Shafeyi 2005, Fazacli & Mazoodi 2006; see Chapter 21 of this book). Other suggested applications are usage as a biosorbent for removal of heavy metal from solutions (Chen et al. 2005) and usage in bioremediation of phenol-contaminated soils and waters (Eggen 1999, Lau et al. 2003, Law et al. 2003). Moreover, directly after the last mushroom harvest, hydrolytic enzymes might be recovered from SMS for industrial applications (Ball & Jackson 1995, Singh et al. 2003, Ko et al. 2005, Rühl et al. 2006), before considering other usages for the SMS.

Wood log cultivation

Mushrooms can be grown on wood logs, providing that they are wood-decaying saprotrophs: *Pleurotus* spp., *Hypsizygus tessulatus* (beech mushroom, buna-shimeji), *Hypsizygus ulmarius* (elm oyster), *Pholiota nameko* (nameko), *Hericium erinaceus* (lion's mane, monkey head mushroom), *Auricularia* spp., *Grifola frondosa* (maitake), *Hypholoma capnoides* (brown-gilled woodlover), *Hypholoma sublateritium* (kuritake, chestnut mushroom) and *Ganoderma lucidum* are such species. The cultivation of mushrooms on wood logs (Fig. 8) is an ancient method (Stamets 1993, Royse 1996). In China, Taiwan, Japan and other East-Asian countries, it is still practised for the industrial production of shiitake. Also in the USA, a small amount of the traded shiitake mushrooms derive from log cultures. Log grown mushrooms give sometimes better quality and fetch higher prices than mushrooms cultivated on sawdust substrate (Brauer et al. 2002, Oei 2003) - although, the latter can have higher protein contents (Aoyagi et al. 1993). Other studies however reported more high-molecular-weight-polysaccharides on log-grown shiitake, including lentinan (Brauer et al. 2002). Cultivation on wood logs does not demand sophisticated technical equipment and is relatively easily done. Therefore, this kind of cultivation is a good choice for hobby-growers or for less-industrialised countries with low wages. For industrial production particularly in Western countries, cultivation on wood logs is

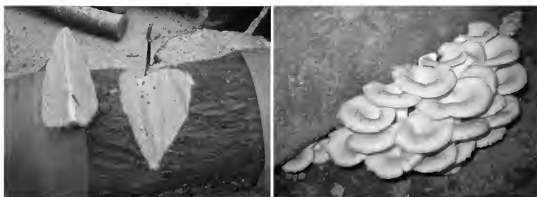


Fig. 8 Preparing logs for fungal inoculation (left) and *Pleurotus citrinopileatus* mushrooms on a beech log

nowadays often replaced by cultivation on synthetic sawdust logs whose nutrient status can be modified specifically to need by adding appropriate supplements (Royse 1996, Royse & Sánchez-Vázquez 2003, Curvetto et al. 2002).

For most mushrooms (*L. edodes*, *Pleurotus* spp., *F. velutipes*, ...), hardwood timber is used for log cultivation, due to the resin contents of softwood species (Stamets 1993). However, *Auricularia* species seems to be less selective and can therefore be grown on both, soft- and hardwood species (Royse 1996, Oei 2003). Before inoculation, logs might be soaked in mineral solutions to optimise the mineral composition of the wood for mushroom production (Queiroz et al. 2004). Besides, all trees should be felled in winter or spring before leafing, when the sugar content in the wood is high. Furthermore, the bark should be kept intact, to prevent desiccation and infections by other organisms (Oei 2003).

For inoculation of logs, different spawning methods can be used: sawdust spawn and spawned wood dowels might be inserted into drilled holes, or wood wedges might be filled with spawned sawdust (Royse 1996, Oei 2003, Fig. 8). The spawned logs should be piled on a stack until the fungi penetrate into the wood. To prevent desiccation, the drilled holes can be sealed with wax, plastic foil or even clay and the stack can be covered. After penetration of the mycelium, the logs should be set up at an appropriate place, where no direct sunlight or reduced humidity is disturbing the colonisation of the whole log. In Japan, shiitake cultivation on wood logs is carried out in forests or forest-like areas under the canopy of trees. For induction of fruiting, the logs can be watered, but it is also possible to wait until the fungus will fruit by itself (Leatham 1982, Raaska 1992, Campbell & Racjan 1999).

Specific mushrooms

Cultivation of *Agaricus bisporus* and relatives on compost

Agaricus species being worldwide the mostly produced mushrooms (see Table 1) are secondary decomposers that can only grow on pre-composted lignocellulosic material. Some manure (chicken or horse dung, urea) has to be added to the lignocellulosic materials in order to obtain a specific C/N-ratio needed for best composting of the unsterile substrate (phase I of substrate preparation). During the subsequent phase II of substrate preparation (pasteurisation and conditioning), the compost is pasteurised to eliminate pests (nematodes, insect eggs and competitive micro-organisms) as well as ammonia, resulting in a better acceptance of the compost by the fungus and a higher mushroom yield. After cooling down to a temperature of 25°C, the substrate is spawned. For mycelial growth in culturing houses, the temperature is kept stable at around 25°C and the CO₂ content should be above 3,000 ppm (Chang & Hayes 1978, Oei 2003). After compost colonisation, a casing layer, consisting of peat, clay, sand and/or already colonised substrate, respectively spent mushroom compost, is added onto the substrate to induce pri-

mordia formation and prevent drying up. Valorising the casing layer with mycelium is called CACing (compost added at casing). The mycelium grows into the casing layer, thereby forming rhizomorphs (strong strands of aggregated mycelium) on which the primordia will develop. Primordia formation in *Agaricus* cultivation is called pinning by the pin-like shape of the young primordia. 2-3 weeks after casing, the mushrooms can be harvested in a 2-4 day lasting flush, recurring in a 7-10 days cycle (Beyer 2003, Walser et al. 2003). The point of harvesting depends to some part on the preferences of the consumer. People in Germany prefer the younger closed or just slightly open white button mushrooms ("button stage" and "cup stage", respectively), while others, e.g. consumers in Great Britain, often want to have mature open caps, in mushroom grower terms called "open flats" (U. Kües, personal observation).

Cultivation of *Pleurotus* species on straw

Pleurotus spp. with *P. ostreatus* (Figs. 6, 7 and 9) as the most common species represent the worldwide second most produced group of mushrooms (Table 1). Other cultivated species are *Pleurotus eryngii* (king oyster; Fig. 9), *Pleurotus sajor-caju* (Indian oyster mushroom), *Pleurotus ferulae* (white elf), *Pleurotus cystidiosus* (maple oyster), *Pleurotus abalonus* (abalone mushroom), *Pleurotus djamor* (pink oyster) and *Pleurotus citrinopileatus* (golden oyster; Fig. 8) (Stamets 1993). *P. ostreatus* grows on a variety of hardwood logs, like on cottonwoods, oaks, alders, maples, aspens, beech, birch, elm, willows, and poplar but with different biological efficiencies (Stamets 2000, Pavlik 2005). In a trial in the Slovak Republic, fresh beech was the best with a biological efficiency of 21% after five years of outdoors log cultivation (Pavlik 2005). A higher biological efficiency of 100% and more in a time of a 2 to 3 month is achieved in industrial cultivation on straw (Fischer 2006; druid Austernpilze, personal communication). Thus, *P. ostreatus* is normally cultivated on straw-

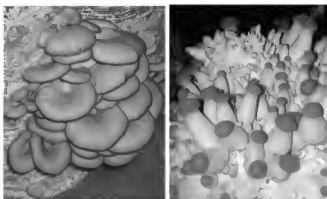


Fig. 9 For comparison of different mushroom shapes: *Pleurotus ostreatus* mushrooms grown on pasteurised straw substrate (left) and *Pleurotus eryngii* fruiting bodies grown on sterilised sawdust substrate (right)

based substrate, of wheat in Europe and North America and of rice in Asia. Several additives are used to improve the quality of the substrate: alfalfa, wheat bran, china reed, soy meal, and others (Lelley 1991, Stamets 1993, Oei 2003). For home purpose and in small mushroom farms, mostly in Asia, the straw is boiled in water to pasteurise it, whereas for larger scale production pre-composting with subsequent heating is usually applied.

After pre-treatment of the straw, the substrate is inoculated usually with grain spawn and filled into suitable containers, e.g. plastic bags, bottles, blocks covered with plastic, and trays (Zadrazil et al. 2004; Fig. 6). The spawned substrate containers are transferred into an incubation room for growth at a temperature of around 25°C (depending on the strain) and subsequent induction of fruiting by lowering the temperature and increasing the humidity (Table 5; Fig. 7). During fruiting, CO₂ has to be controlled, since it affects the shape of the stipe. High CO₂ levels will increase the stipe length. In cultivation of *P. ostreatus* (Fig. 7 and 8), a low CO₂ concentration (< 700 ppm) is necessary during fruiting body formation to suppress stipe elongation (Stamets 1993, Oei 2003). In contrast, in *P. eryngii* a long stipe is expected by the consumers (Fig. 8), which can be realised by reduced fresh air supply (C. Schöpper & M. Rühl, unpublished observation).

Cultivation of *Lentinula edodes*, *Pholita nameko* and other white-rots in sawdust culture

Worldwide third in cultivation (Table 2), *L. edodes* is most popular in Asia, and its popularity as an edible mushroom with health-caring effects is also increasing in Europe (Campbell & Racjan 1999). Growth substrate compositions usually base on sawdust to which wheat or rice bran, cane sugar, or molasse might be added as extra carbohydrate source, and urea or ammonium chloride as N source (Stamets 1993, Kalberer 2000, Oei 2003). Whilst substrate recipes are found in the literature, strong efforts are still made to find optimal media for *L. edodes* cultivation - as well as for cultivation of other species such as *G. frondosa*, *H. erinaceus*, *P. nameko*, and several *Pleurotus* species, growing on sawdust based substrates (Fig. 9 and 10).

In Europe, shiitake is mostly cultivated in polypropylene bags and in Japan sometimes in bottles which can withstand the high temperatures during thermal steam sterilisation. Substrate containers can be filled manually or automatically (Fig. 6). After sterilisation and cooling down, spawning is performed either by putting grain spawn onto the substrate, by plugging in wooden mycelium-infested sticks, or by liquid spawn from fermenter cultures (Oei 2003). During subsequent growth, air exchange with the environment is ensured by a cotton plug closing bags and bottles or by an integrated filter (Table 3). Fungal colonisation of the sawdust substrate takes 4-6 weeks, at the end of which the fungi will form under elevated CO₂ concentrations a thick white coat with mycelial aggregations ("bumps") on the substrate surface (Oei 2003; Fig. 9). After removal of the plastic

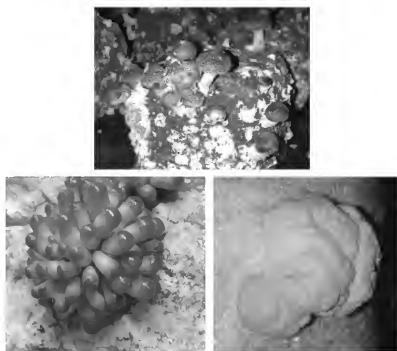


Fig. 10 Fruiting bodies of *Lentinula edodes* (top), *Pholiota nameko* (left) and *Hericium erinaceus* (right) grown on beech sawdust substrates

bags or the cotton plug enabling aeration, the surface coat will turn reddish-brown pigmentation phase) which prevents desiccation and contamination (Lelley 1991). Subsequently, primordia formation is induced by soaking and further cultivation at (high air humidity, low temperature, and a low CO₂ level. For fruiting body maturation, the relative humidity should be lowered and an appropriate aeration maintained to avoid high CO₂ concentrations (Table 5). After harvest, increasing the temperature starts the regeneration phase for the mycelium to acquire new nutrients for a new round of production.

Production of other white rot mushrooms on sawdust is similarly performed than shiitake cultivation but this occurs without formation of a pigmented coat by the fungus and a soaking step performed by the mushroom grower for induction of fruiting (for details on cultivation on sawdust of other species see Lelley 1991, Stamets 1993, Oei 2003).

Conclusions

Mushroom cultivation is an elegant way to add value to lignocellulosic wastes from agriculture and forestry. Worldwide, the culinary interest in edible fungi is steadily increasing and with it, the market for mushrooms and the number of cultured species. Cultivation of some species is well established whilst for others, better growth conditions are still searched for. Breeding new strains can help to

better adapt the mushrooms to specific substrates and specific culture conditions and maintains the genetic qualities of species. Modern molecular techniques and the genetic knowledge obtained by a few selected model organisms [e.g. *C. cinerea* (see Chapter 23 of this book) and the pathogenic white-rot *S. commune*] can assist in breeding to improve production and mushroom properties (Kothe 2001).

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23. Mushroom Biology and Genetics

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Introduction

Worldwide, a large range of mushrooms are collected as non-timber forest products for human consumption and/or health purposes (Boa 2004). Total harvests of fruiting bodies from the forests are however difficult to estimate. According to interviews with mushroom pickers and brokers, the economic value of collected Boletes, Chanterelles and Pine Mushrooms in Washington State, Oregon and Idaho in 1992 comprised US \$14 million (Schlosser & Blatner 1995). Following recorded exports to Japan, the value of the Canadian 1994 harvest of Pine Mushrooms added up to at least US \$18.5 million (de Geus & Berch 1997). The global trade in wild edible fungi has been estimated at US \$2 billion (Hall et al. 2003a). The complete value of mushrooms as non-timber forest products is however much higher, since

large amounts of fruiting bodies collected for personal use are left aside in this number (Boa 2004). On occasions, the total value of mushroom as non-timber forest products may reach in a given forest that of the timber - usually, it is lower than that of the timber. Reasons for this are the relative low and variable yields of mushrooms at short seasons and the relative high effort needed to find and collect them (Alexander et al. 2002). Moreover, harvests of many mycorrhizal mushrooms have declined dramatically over the past hundred years (Yun & Hall 2004). As a consequence, certain forest mushrooms belong to the most expensive foodstuff (Kües & Liu 2000, Boa 2004, Mello et al. 2006, Yamada, A. et al. 2006).

Harvests of wild mushrooms are influenced by the changing climatic conditions with from year to year varying results. Furthermore, measures in forest management can have effects on mushroom yields whilst effects of mushroom harvesting are differentially estimated (Kües & Liu 2000, Pilz & Molina 2002, Boa 2004, Luoma et al. 2004, Pilz et al. 2004, 2006, Ogaya & Penuelas 2005, Sinha & Brault 2005, Egli et al. 2006). For reliable mushroom yields independent of seasonal climate conditions and other factors, commercial production processes are essential. The number of cultivable species increased over the years but all these are saprotrophs (Boa 2004; see Chapter 22 of this book). In contrast, many of the mushrooms collected from the forests are mycorrhizal. Obviously, such symbiotic species require being associated with their hosts in order to fruit. Where tried, mycorrhizal species resisted so far mushroom production in culture with a few exceptions where a fungus was cultivated together with its host. At the best, artificial inoculation of roots lead to increased mushroom yields at natural sites (Ayer & Egli 1997, Danell & Camacho 1997, Kües & Liu 2000, Yamada et al. 2001, Hall et al. 2003b, Boa 2004, Salerno & Perini 2004, Yun & Hall 2004, Bonet et al. 2006). Nevertheless of success with some fungi, propagation by in situ root inoculation and subsequent transplantation is so far not applicable to all species (Hall et al. 2003b, Guerin-Laguette et al. 2005).

For most species, the biology of fruiting body production is not at all or only poorly described. Generally, fruiting body development is environmentally regulated by a combination of signals such as the overall nutrient availability, the C to N relationship, temperature, light, humidity, aeration and CO₂ concentrations (Kües & Liu 2000, Peter et al. 2001, Kües et al. 2004, Wösten & Wessels 2006; see Chapter 22 of this book). Mycorrhizal fungi in addition may receive signals from their hosts (Smith et al. 1994, Bücking & Heyser 2001, 2003, Nehls et al. 2001, Yamada et al. 2001, Wu et al. 2002). The monographs by Cléménçon (1997, 2004) compile on a subset of basidiomycetes what is available on the cytology of mushroom development. Understanding the physiology and cytology of fruiting body development is expected to contribute to establishing and improving cultural conditions in commercial mushroom production (Chiu et al. 2000, Kües et al. 2004; see Chapter 22 of this book) and to help to sustain natural production of mycorrhizal species that remain to be collected from the forests (Yun & Hall 2004, Ogaya & Penuelas 2005). Breeding of edible and pharmaceutical mushrooms targets at obtaining efficient, reliable and

easy to manage strains for economical production of high quality fruiting bodies (Kothe 2001, Kitamoto 2006). Such breeding is based on selection of genetic variants that produced fruiting bodies of desirable quality and appearance from wild genetic resources (Stoop & Mooibroek 1999, Chiu et al. 2000, Kitamoto 2006).

Studies for better understanding of fruiting body development require genetically accessible sexual systems. By the ease to obtain mushrooms in culture, the dung fungus *Coprinopsis cinerea* (formerly called *Coprinus cinereus*; Redhead et al. 2001; common name: inky cap fungus) is used by now for over hundred years as the model species to unravel the basic biological principles in fruiting body development of higher basidiomycetes (Brefeld 1877, Buller 1924, Kües 2000, Kamada 2002, Fischer & Kües 2003, Kües et al. 2004). Unlike other basidiomycetes, the species is well accessible by both classical and molecular genetics (Binninger et al. 1986, Granado et al. 1997, Walser et al. 2001).

Fruiting body development of *Coprinopsis cinerea*

C. cinerea has a standard basidiomycete life cycle starting with the infertile monokaryons that germinate from the sexual haploid basidiospores. Monokaryons are characterised by hyphae with simple septa and usually one haploid nucleus in the hyphal cells. Mating compatible monokaryons (those that have a different mating type) fuse in order to form the fertile dikaryon. This is a secondary mycelium carrying in all hyphal cells one haploid nucleus from each parental monokaryon and clamp cells as specialised structures at the hyphal septa (see Fig. 2 and 3 in Chapter 22 of this book). Fruiting bodies are easily obtained from dikaryons in culture. Sexual spore formation occurs on the basidia, specific cells located on the surface of the mushroom gills. In the basidia, the two parental types of haploid nuclei fuse (karyogamy) to give a diploid nucleus with two sets of chromosomes. The two reduction divisions of meiosis directly follow the karyogamy with the final result of four haploid nuclei. Each one of these nuclei migrates into one of the four different basidiospores formed externally on the basidium, which completes the fungal life cycle. In total, it takes only two weeks for *C. cinerea* to go through the whole life cycle (Bensaude 1918, Buller 1924, Kües 2000, Kües et al. 2002a; see Chapter 22 in this book).

Whilst growth of the vegetative mycelium of *C. cinerea* occurs best at 37°C, fruiting body development takes place only at 25–28°C. Fruiting requires day-night changes (e.g. a 12 h light/12 h dark regime or 16 h light/8 h dark regime) and >80% humidity (Granado et al. 1997, Walser et al. 2001). In total, six days are needed from the first step of fruiting body development (primary hyphal knot formation) over tissue formation in the fruiting body primordia to fruiting body maturation. Because of autolysis typical for the coprinoid mushrooms, fruiting bodies of *C. cinerea* are only short-lived. Being fully established after midnight in the early morning hours, they usually are already collapsed at midday of the next day (Boulianne et al. 2000, Kües 2000, Walser et al. 2003; Fig. 1). In case environmental conditions change

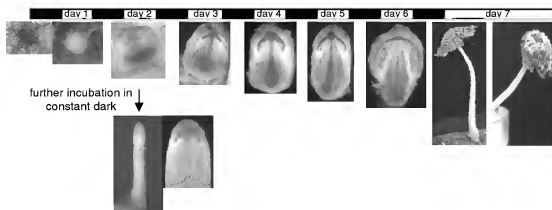


Fig. 1 The course of fruiting body development in *Coprinopsis cinerea* over the time on an artificial agar medium (Granado et al. 1997). After mycelial growth at 37  C in the dark, cultures were transferred at the beginning of the light phase of day 0 into a 12 h dark/12 h light regime at 28  C. Night periods are shown in the bar above the photos by black boxes, day periods by white boxes. Fruiting body development starts in the dark by intense branching at single or a few hyphae of the vegetative mycelium in order to form loose hyphal aggregates (primary hyphal knots). Upon receipt of a light signal, hyphal cells aggregate firmly into small round bodies (secondary hyphal knots; day 1). Within these bodies, tissue development of stipe and cap starts (day 2). When kept further in the dark, long structures (dark stipes or etiolated stipes) form with extended stipes and underdeveloped caps. To progress properly in tissue formation in the fruiting body primordia, three dark-light changes are required before another light signal induces karyogamy within the basidia of the fully established primordia (day 5). In parallel to karyogamy (start of light phase at day 6), subsequent meiosis and spore formation, the stipe elongates and the cap opens (afternoon, night hours of day 6). Some of the basidiospores might be ejected from the gills of the opened mushroom. However, soon after maturation the cap autolyse (day 7 late morning up to midday) in order to release the majority of basidiospores in liquid droplets (Iten 1970, Moore et al. 1979, Walser et al. 2003, K  es et al. 2004). With the exception of the autolysed fruiting body, all developmental stages were photographed at the switch from dark to light (hour 8 on the 24 h clock scale)

during any of the earlier stages, mushroom development will arrest. Most interesting is the effect of too little light. Instead of normal mushrooms, so-called dark stipes or etiolated stipes with underdeveloped caps and strong, elongated stipes are formed that will not autolyse (Lu 1974, Fig. 1). This property is made use of by small farmers in Thailand in the cultivation of *C. cinerea* mushrooms on agricultural wastes. Resulting etiolated stipes are harvested, shortly boiled in water and pickled in salt for sale (Fig. 2). Similarly, by the constant addition of new waste material onto the compost heaps in the sisal plantations in Tanzania, young *C. cinerea* primordia are kept in dark conditions and are thus hindered from normal development. Etiolated stipes are



Fig. 2 A commercial dikaryotic strain of Hed-Cone-Noy (*Coprinopsis cinerea*) from Thailand grows and fruits at 28°C on wheat straw (photos left). Hed-Cone-Noy pickled in a 50% salt solution from a Thai food store (photos right; kindly supplied by Jiraphant Supadit). Note that the pickled Hed-Cone-Noy are immature primordia as obtained under exclusion of light. The inscription on the glass states as values for nutrition low fat, no cholesterol, high mineral content, and potassium and promises consumers decrease of blood pressure, protection against cancer cells by selenium, quickening the appetite by glutamic acid, and decrease of blood sugar as a preventive action in diabetes

grubbed out by plantation workers and fried as a welcome tasty addition to their lunches (Härkönen et al. 1993).

Genetic access of fruiting body development

As stated above, fruiting body development of *C. cinerea* occurs in nature on the dikaryons. Dikaryon formation is genetically controlled by the two mating type loci *A* and *B*. The *A* mating type genes encode homeodomain transcription factors, proteins that control expression of downstream genes in sexual development. The *B* mating type genes encode pheromones and pheromone receptors, mediating signalling between cells and/or nuclei of different mating type. To form and maintain a dikaryotic mycelium, both *A* and *B* mating type genes of different specificity are needed (Casselton & Olesnický 1998, Hiscock & Kües 1999, Brown & Casselton 2001, Casselton & Challen 2006, Casselton & Kües 2007). Cloned mating type genes were transformed into *C. cinerea* monokaryons. Next to eliciting typical features of the dikaryotic mycelium (e.g. clamps cells and clamp cell fusion; Kües et al. 1992, 1994a,c, Badalyan et al. 2004), the *A* mating type genes were shown to induce hyphal knot and primordia development (Tyman et al. 1992, Kües et al. 1998) and the *B* mating type genes to support the *A* mating type genes in initiation of fruiting body development and to act in fruiting body maturation at the stage of karyogamy (Kües et al. 2002b). Therefore not surprisingly, certain mutations in the mating type loci (Kües et al. 1994b, Pardo et al. 1996, Olesnický et al. 1999, Srivilai et al. 2006b) make fruiting body development independent from dikaryon formation (Swamy et al. 1984). Basidiospores with such mutations germinate into a self-compatible, fertile mycelium with a dikaryon-like

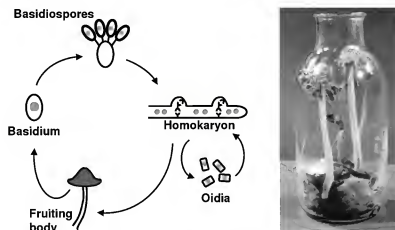


Fig. 3 Simplified life cycle of the self-compatible *Coprinopsis cinerea* homokaryon AmutBmut. Fruiting bodies of homokaryon AmutBmut formed on sterilised horse dung in the laboratory are shown at the right (photo: Markus Aebi)

appearance: it has clamp cells at the hyphal septa and often two haploid nuclei in the hyphal cells – since these nuclei are genetic identical, the self-compatible mycelium is called a homokaryon: Also like the dikaryon, under appropriate environmental conditions fruiting bodies develop on the homokaryotic mycelium (Fig. 3).

Self-compatible homokaryons of *C. cinerea* offer a unique genetic opportunity to study fruiting body development. On the dikaryon, dominant mutations in fruiting body development can easily be found whereas recessive mutations will be overlooked unless a respective mutation is present in both nuclei of the mycelium (Moore 1981). This situation is different in the self-compatible homokaryons. Recessive mutations in fruiting body development result in apparent phenotypes (Lu et al. 2003, Liu et al. 2006, Srivilai et al. 2006a; Fig. 4). Therefore, self-compatible homokaryons are appointed in mutagenesis programmes (Granado et al. 1997, Cummings et al. 1999, Muraguchi et al. 1999, Kües et al. unpublished). Like monokaryons, the self-compatible homokaryons are able to produce abundant asexual, single-celled uninucleate spores (called oidia; Fig. 3) with a haploid nucleus. However, oidia production is not constitutive as in monokaryons but light induced as is asexual sporulation in dikaryons (Polak et al. 1997, 2001, Kertesz-Chaloupková et al. 1998, Kües et al. 2002a, Fischer & Kües 2006). For mutagenesis, oidia can be treated with chemicals or UV-light or they might be transformed with external DNA that integrates into the genome, thereby interrupting the genes at the places of integration (Walser et al. 2001). Screening for mutations showed that defects in fruiting body development occur relatively often - the mutation frequency can rise above 1% - suggesting that many different genes contribute to the complex process of fruiting body and sexual basidiospore development (Takemaru & Kamada 1969, 1970, 1972, Granado et al. 1997, Cummings et al. 1999, Muraguchi et al. 1999, Kües et al. unpublished). Cloning

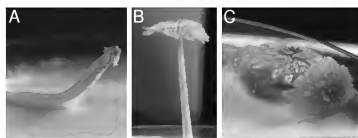


Fig. 4 Mutants in fruiting body development of self-compatible homokaryons. A. Strain PUK1 has a *dst1* defect and thus forms etiolated stipes also in light (Terashima et al. 2005). B. Mutant OU2 with a yet uncharacterised mutation in basidiospore formation (*bad*) forms white mushrooms without spores (Srivilai et al. 2006a). C. UFO1 is a mutant with a defect in stipe elongation (*eln*) and forms therefore dumpy mushrooms. The fruiting body cap is white due to an additional *bad* mutation (Srivilai et al. 2006a)

of genes by mutant complementation supports the idea that various types of genes contribute to fruiting.

Since work intensive, from mutant analysis in *C. anerea* only a handful of genes have so far been identified that are engaged in fruiting body development. A yet uncharacterised gene *pen1* has been cloned that is essential for primary hyphal knot formation (Clergeot et al. 2003 and unpublished results). Gene *qst1* is a gene required for secondary hyphal knot formation. It encodes a cyclopropane fatty acid synthase belonging to the family of C-methyltransferases that adds methylene-groups from S-adenosyl-methionine molecules across C-double bonds in membrane-localised lipids in order to form a cyclopropane ring (Loos 2001, Pemmasani et al. 2005, Liu et al. 2006). Gene *ich1* acts in cap tissue formation and genes *eln2* and *eln3* in stipe elongation. *ich1* encodes a potential O-methyltransferase, *eln2* a cytochrome P450 and *eln3* a potential glycosyltransferase probably involved in cell wall biogenesis (Muraguchi & Kamada 1998, 2000, Kües 2000, Arima et al. 2004). Defects in the *dst1* gene lead to the dark-stipe phenotype and this gene encodes a putative blue light receptor of the white-collar 1 type (Terashima et al. 2005; Fig. 4A). White cap mutants (Fig. 4B) lacking any basidiospores helped to clone the gene *spo11* for a type II topoisomerase active in meiosis (Cummings et al. 1999, Celerin et al. 2000). Two further genes have been identified by mutant analysis that act in the *A* mating type pathway. A defect in gene *cb1* blocks clamp cell formation and fruiting body formation in self-compatible homokaryons (Inada et al. 2001). In contrast, mutations in gene *pac1* that encodes a MHG-box transcription factor allow fruiting body development on the monokaryon (Murata et al. 1998a,b).

DNA transformation has a crucial role in mutant analysis: wildtype genomic DNA cloned in suitable *Escherichia coli* plasmid constructs [often phage λ -related cosmids that can take up foreign DNA in length of up to 40 kb stretching over with several genes (Bottoli et al. 1999)] is introduced into the mutants in order to identify

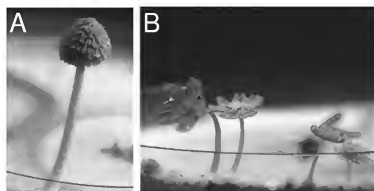


Fig. 5 A. The self-compatible *ds1* mutant forms wildtype mushrooms, when transformed with cosmid 40-5A that carries a *ds1* wildtype gene (Liu et al. 2006). B. A wildtype dikaryon carrying a *ras*^{Val19} copy forms small "Bonsai" mushrooms (P. Srivilai et al. unpublished)

the defective genes by restoration of the wildtype phenotype (e.g. see Liu et al. 2006; Fig. 5A). However, with dominant phenotypes it is also possible to use wild-type strains in transformations of developmentally regulated genes under control of foreign promoters (Inada et al. 2001; see below). Analysis of dominant mutant genes by transformation in wildtype strains is also possible. *ras* genes, encoding small GTPases functioning in signal transduction pathways, are genes of which mutant versions with dominant, constitutive active phenotypes have been described in various organisms. Normally in cells, Ras GTPases switch between two physiological stages, an active form where GTP is bound to the protein and an inactive form without GTP. By mutation constitutively activated Ras proteins have an amino acid substitution in the GTP binding site that causes GTP to permanently bind to the proteins (Bourne et al. 1991, Paduch et al. 2001, Weeks & Spiegelman 2003). An analogous mutant (*ras*^{Val19}) has been constructed by *in vitro* mutagenesis from a cloned *ras* gene of *C. anerea* (Bottoli 2001). When introduced into *C. anerea* wildtype strains, multiple phenotypes are observed including alterations in tissue formation within fruiting bodies and defects in stipe elongation (Bottoli 2001, Srivilai 2006, Srivilai et al. 2006c; Fig. 5B). These multiple phenotypes very much resemble those described in a *Schizophyllum commune* mutant lacking the gene *gap1* for the Ras GTPase-activating protein that promotes hydrolysis of Ras-bound GTP to GDP and thereby inactivates the protein (Schubert et al. 2006). Both the *ras*^{Val19} transformed dikaryons of *C. anerea* and the *gap1* defective dikaryons of *S. commune* show defects in morphological features regulated by the *B* mating type pathway (Schubert et al. 2006, P. Srivilai et al. unpublished). By co-transformation, it is possible to introduce simultaneously two or more genes into *C. anerea* strains (Kües et al. 2001b). Co-transformation of *ras*^{Val19} and *B* mating type genes into suitable *C. anerea* monokaryons could become an interesting experiment in the future.

Other than by mutant analysis, two fruiting body-specific genes (*ggl1*, *ggl2*) for galectins [belonging to a β -galactoside binding family of lectins (Walser et al. 2004)] have been identified via the expressed proteins (Charlton et al. 1992, Cooper et al. 1997). These proteins are specifically expressed in the outer cap and stipe tissues (Boulianne et al. 2000), together with ligands of likely a lipid nature (Walser et al. 2004, 2005). The proteins have been suggested to contribute to hyphal aggregation (Boulianne et al. 2000). They are however not essential as recently shown by gene inactivation in a self-compatible homokaryon through the posttranscriptional acting RNAi technique that leads to degradation of gene transcripts (Wälti et al. 2006). Establishing this technique in *C. cinerea* in various laboratories (Namekawa et al. 2005, Heneghan et al. 2006, Wälti et al. 2006) is a great achievement for analysis of those genes for which mutants are not available. The gene for the LIM15/DCM1 recombinase has been inactivated in a self-compatible homokaryon with the result of an abnormal chromosomal pairing in meiosis, a highly reduced basidiospore number and viability (Namekawa et al. 2005). Classical knocking-out of genes via exchange by homologous recombination of wildtype genes in the fungal genome by non-functional gene copies with an inserted transformation marker gene has been attempted before in *C. cinerea*. In cases where gene knock-outs were obtained; the frequency was low: 1% and less of all analysed transformants (Binninger et al. 1991, Pardo 1995, Kües et al. 2004, Walser 2004). With the complete *C. cinerea* genome sequence made available to the public by the Broad Institute (http://www.broad.harvard.edu/annotation/fungi/coprinus_cinereus/background.html), that allows identifying genes of interest by their sequences, functional gene activation techniques have gained major importance.

To us, two groups of genes within the *C. cinerea* genome were of primary importance, those for hydrophobins since they were shown *in vitro* to interact with the mushroom galectins [work by P.J. Walser presented in Velagapudi (2006)], and those for laccases whose functions in fruiting are a matter of dispute (Kües & Liu 2000, Wösten & Wessels 2006). Hydrophobins are small secreted proteins with 8 conserved cysteine residues. They cover aerial hyphae in order to provide them a hydrophobic surface needed for growth in the air and they line air channels in fruiting bodies in order to prevent them from water logging (Wösten 2001, Walser et al. 2003, Linder et al. 2005, Peddireddi et al. 2006, Velagapudi 2006). Recent results in the ascomycete *Aspergillus oryzae* further suggest that hydrophobins recruit other proteins to the fungal surface (Takahashi et al. 2005). In the *C. cinerea* genome, as many as 34 different hydrophobin genes were found by sequence analysis and many of them are expressed in the fruiting bodies. Very few are, however, specific to the fruiting body (Velagapudi et al. 2005a,b, Velagapudi 2006). With a total of 17 different genes, laccase genes are also manifold present in the *C. cinerea* genome (Hoegger et al. 2004, Kilaru et al. 2006a). Also many of these genes are transcribed in the fruiting bodies, particularly in the cap during formation of the black basidiospores (M. Navarro-González et al. 2005). Specific

fungal laccases have been implicated in pigment formation (Langfelder et al. 2003, Youngchim et al. 2004, Walton et al. 2005) but phylogenetic analysis of the *C. cinerea* laccases do not support such a function for any of the enzymes (Hoegger et al. 2006). It will be a major effort to individually inactivate the various hydrophobin and laccase genes in *C. cinerea* to test their specific functions in the fruiting bodies. The simultaneous silencing of the two galectin genes *gl1* and *gl2* by the RNAi technique (Wälti et al. 2006) gives hope that this could also become the case at least for the closer related hydrophobin genes, respectively laccase genes.

Further important recent achievements for studies of genes acting in fruiting body development are the availability of regulated and constitutive promoters and the availability of reporter genes. Laccase genes have been shown to be versatile reporter genes for studying activities of fruiting body specific promoters. Promoter activities are recorded by enzymatic conversion of colourless substrates into coloured products (Velagapudi 2006, Velagapudi et al. 2006). Another functional reporter in *C. cinerea* is the green fluorescent protein GFP from the jellyfish *Aequorea victoria* (Burns et al. 2005). The promoters of the galectin genes can be used to express genes specifically in the early stages of fruiting body development (Bertosso et al. 2004, Velagapudi et al. 2006). Various constitutive promoters - with the *Agaricus bisporus* *gpdII* promoter being most effective - might be used to express fruiting body-specific genes within the vegetative mycelium, for example to obtain larger amounts of proteins for their characterisation (Burns et al. 2005, Kilaru et al. 2006b,c). Constitutive expression of developmental regulator genes can be of interest to confirm suspected functional roles. For instance, gene *clp1* induces under such conditions clamp cell formation in monokaryons confirming an operative position in sexual development downstream of the *A* mating type genes (Inada et al. 2001).

How can *Coprinopsis cinerea* research contribute to the understanding of mushroom formation in other species?

A number of interesting gene functions have now been described in fruiting body development of *C. cinerea* (see above), but their interrelations are far from clear. Certainly, they are just first isolated parts of a large puzzle and it will require time to add more and more pieces to the picture. Large scale gene expression analysis (sequencing of EST libraries, micro-array studies, differential display techniques, proteomic approaches, etc.) are means to quickly identify bulks of genes that are active at specific developmental stages. For *C. cinerea*, over 15,000 ESTs (expressed sequence tags, denoting transcripts from active genes) are currently deposited in the NCBI (National Center for Biotechnology Information) sequence database. Over 2800 of these are from the fruiting body cap (<http://www.ncbi.nlm.nih.gov>). In other basidiomycete species (*A. bisporus*, *Flammulina velutipes*, *Lentinula edodes*, *Pleurotus ostreatus*, *S. commune*), large scale expression

studies have also been performed and/or differentially expressed genes identified for stages of fruiting body development (Mulder & Wessels 1986, de Groot et al. 1997, Leung et al. 2000, Ospina-Giraldo et al. 2000, Eastwood et al. 2001, Lee et al. 2002, Hirano et al. 2004, Miyazaki et al. 2005, Sunagawa & Magae 2005, Park et al. 2006, Yamada, M. et al. 2006). Comparison between species might identify those functions which are globally important in mushroom development.

Although for all important edible species DNA transformation systems have now been described, compared to *C. cinerea* transformation methods (Binnering et al. 1987, Granado et al. 1997), these are very inefficient and less reliable since more difficult to perform (van de Rhee et al. 1996, Chen et al. 2000, Honda et al. 2000, Irie et al. 2001, 2003, Kikosch et al. 2001, Sunegawa & Magae 2002, Kuo et al. 2004, Burns et al. 2005, Li et al. 2006). Studying gene functions extensively first in easily to obtain *C. cinerea* transformants, thereby defining their exact roles in development, can drastically reduce the workload when later confirming gene functions in the other, less approachable species.

It is in addition possible to transform candidate genes from other species into the characterised *C. cinerea* mutants to check conservation of functions. Currently, only mating type genes from *Coprinellus disseminatus* (formerly *Coprinus disseminatus*), *Coprinopsis scabicola* (formerly *Coprinus bilanatus*) and *C. cinerea* under control of their native promoters have unambiguously been shown to work in heterologous higher basidiomycetes (Challen et al. 1993, Kües et al. 2001a, James et al. 2006, Srivilai 2006). Several other basidiomycete promoters have been found to work across species borders (Alves et al. 2004, Burns et al. 2005, Kilaru et al. 2006b,c, Rekangalt et al. 2004, 2007). Promoters of heterologous genes are therefore not expected to cause principle problems for expression in *C. cinerea*. However, fruiting body-specific promoters from *L. edodes* and *S. commune* are expressed in the vegetative mycelium of monokaryons of *C. cinerea* (Kilaru et al. 2006b, P. Srivilai, unpublished observation) and *Phanerochaete chrysosporium* (Alves et al. 2006) whereas the fruiting body-specific *glt1* promoter from *C. cinerea* is expressed in the vegetative mycelium of *Hebeloma cylindrosporum* (Rekangalt et al. 2004, 2007). This different behaviour of the promoters might not necessarily be due to that transcription factors react differentially in the species. An altered global sequence environment in which the promoters are embedded in the cloning vectors could also explain the results. This idea is supported by experiments with the *gfs1* and *pkn1* mutants of *C. cinerea*. For fully complementation of the *gfs1* and *pkn1* defects, it was not sufficient to transform just the wildtype genes with their promoters into the mutants. Instead, larger DNA fragments were required containing also the natural neighbouring genes (Liu et al. 2006, P.-H. Clergeot et al. unpublished). Inactivating the *gfs1* neighbouring genes by small internal deletions on the DNA fragments used in transformation did not derogate the complementation results. This suggests that the sensitive factor for correct expression is the global structure of the chromosome at the places where the genes naturally reside (Liu et al. 2006). In

mutant complementation experiments with heterologous genes in *C. cinerea* fruiting mutants, exchanges of native promoters with fruiting-body specific promoters on larger DNA fragments might therefore be required.

Transformation of mating type genes and the constitutive active *ras³/alt⁹* mutant allele into different *C. cinerea* monokaryons revealed that developmental reactions can differ in strength and occurrence, depending on the genetic background of the respective strains (Kües et al. 1998, 2002b, Bottoli 2001, Srivilai 2006).

Firstly, this is interesting since it indicates that in nature there is much flexibility in the system due to the normal dikaryotic state of the fruiting mycelium (Moore 1981, Kües 2002). Breeding programmes for highly efficient strains in *P. ostreatus* showed that mushrooms have many genetic loci scattered over the whole genome which contribute to the fruiting body productivity of the strains (QTLs, quantitative trait loci; Larraya et al. 2003). Such QTLs also appear to exist in *C. cinerea* (Srivilai 2006).

Secondly, the results from transformations of mating type genes and *ras³/alt⁹* in different *C. cinerea* monokaryons indicate that observations from transformations have carefully to be evaluated in the best suitable strains. Such ideal strains might be obtained by genetic crosses under appropriate selection for best fruiters with healthy mycelial growth (Kitamoto 2006). In classical crosses of distantly related *C. cinerea* strains, large proportions of progenies will perform poorly in growth and/or in fruiting body development (Liu et al. 1999, Srivilai 2006, P. Srivilai et al. in preparation). Since positively selected for their ability to form fruiting bodies without a prior need of mating, self-compatible haploid homokaryons present strains with an expected good-quality pool of fruiting genes (Swamy et al. 1984). By repeated backcrosses against strong, fruiting-proficient, self-compatible homokaryons, monokaryons co-isogenic to these homokaryons have been obtained carrying such good-quality pool of fruiting genes (Liu et al. 2006, Srivilai 2006).

Conclusions

Little is so far understood about the physiological and genetic backgrounds of the very complex differentiation processes in mushroom development in the higher basidiomycetes. By the easy access through classical and molecular genetics, *C. cinerea* is the preferred species to study fruiting body development. The fungus forms mushrooms with cap and stipe. In *C. cinerea*, large collections of mutants are available that can help to unravel the basics of the fruiting processes.

The genomes of the saprotroph *C. cinerea* (http://www.broad.harvard.edu/annotation/fungi/coprinus_cinereus/background.html), the white-rot *P. chrysosporium* (Martinez et al. 2004, Vanden Wymelenberg et al. 2006) and the ectomycorrhizal fungus *Laccaria bicolor* (<http://www.jgi.doe.gov/sequencing/DOEmicrobes2006.html>) are released to the public. Genome sequencing of the wood-colonising species *S. commune* [the other model of higher basidiomycetes for

studying developmental processes which has fan-shaped fruiting bodies (Kothe 2001, Wösten & Wessels 2006)) is ongoing at JGI (DOE Joint Genome Institute; <http://www.jgi.doe.gov/sequencing/DOEMicrobes2006.html>). In addition, sequencing of the genome of the brown-rot *Postia placenta* has been accomplished and the JGI has agreed to sequence other economical and ecological important higher basidiomycetes (the edible white-rot *P. ostreatus*, the edible saprotrophic compost fungus *A. bisporus*, the pathogenic white-rot *Heterobasidion annosum*, the brown-rot *Serpula lacrymans*, and the ectomycorrhizal species *Paxillus involutus*). Soon, we will have a vast amount of sequence data that will help to perform comparative studies on genes acting in fruiting body development in order to better understand the process also in terms of sustainable fruiting body production by the mushroom industry and possibly in the forests, too.

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24. Wood and other Plant Fibres in the Production of Peat Substitutes and Pot Plant Containers

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Introduction

Conventional plant growth substrates used in the production of horticultural crops and ornamentals in greenhouses, homes, gardens, and nurseries are predominantly based on peat. Physical and chemical properties of peat (lightweight long-lasting material, water holding capacity, porosity and aeration properties, mineral leaching, nutrient retention, pH range, etc.) well satisfy needs for plant growth when combined in commercial peat formulations with suitable fertilisers (McBriertry et al. 1996, Macz et al. 2001, Clemmensen 2004, Smith et al. 2004, Agner & Schenk 2005).

Peat however is a non-readily renewable resource. Peatland development is a long-term process lasting for millennia (Halsey et al. 1989, Miner & Ketterling 2003, Holden 2005). In Europe, upland moors were generated 12,000 years ago at the end of the ice-age. In the last 200 years, upland moors have been dried up to large extends by peat mining and conversion to land for agricultural and forestry use. Many countries in Western Europe have lost more than 90% of their peatland heritage. The most drastic losses are encountered in Finland, the Netherlands, Estonia, Denmark, and the UK (Bragg & Lindsay 2003). Nonetheless, the last European peat bogs are still exploited as an energy source and for horticultural purposes, particularly in Ireland and in the Baltic states (Cruickshank et al. 1995, Organo et al. 2005, Paist et al. 2005, Wicker 2005). In North America, the Canadian states Quebec, New Brunswick, Manitoba and Alberta are the primary peat-producing regions (Warner & Buteau 2000).

Harvesting of peat includes drainage of moors which influences groundwater levels by reduction of 1 to 3 cm per year. In addition, nutrients in form of carbon and nitrogen can be lost by drainage. Peatlands store between one-third and one-half of the world's soil carbon and they play an important role in moderating the global climate. Drying of peatland and usage of the organic peat also gives rise to emissions of climate gases (5 million tons/year; in particular carbon dioxide, methane and nitrous oxide) and to global warming (Gorham 1991, Augustin et al. 1998, Bragg & Lindsay 2003, Huttunen et al. 2003, Cleary et al. 2005, Holden 2005, Zedler & Kercher 2005). The extraordinary ecosystem moor shows a very specific biodiversity and is a hideaway for many rare organisms (Calme et al. 2002, Locky et al. 2005, Whitehouse & Bayley 2005, Zedler & Kercher 2005). Peat mining negatively affects the ecological communities of these unique habitats and endangers species confined to these wet, nutrient deprived biotops (Soro et al. 1999, Campbell et al. 2003, Mazerolle 2003, Lachance et al. 2005). Moreover with peat mining, chronological paleoenvironmental records hidden in the ancient peat profiles irrecoverable get lost (Chapman et al. 2003).

Peatland has some potential for natural regeneration but sustained yield of peat in short time is limited due to an inherent slow growth (Girard et al. 2002, Chapman et al. 2003, Poulin et al. 2005). For example in Finland, peat growth is estimated 85 times slower than peat use (Schilstra 2001). Nowadays, efforts are made in several parts of the world to restore fens and bogs after peat mining. Since the water chemistry in post-harvest and undisturbed peatland can significantly differ particularly in pH and concentrations of ammonia and nitrate, flourishing of ombrotrophic bog species (wetland species almost exclusively depending on water from precipitation) in post-harvest peatland can become a problem (Wind-Mulder and Vitt 2000). Rewetting measures and seeding with donor diaspore material (agents for plant dispersal such as seeds, spores in case of bryophytes, etc.) help in restoration. Vegetation patterns following restoration resemble those of natural peatlands but the man-made biotops are too young to

fully copy the characteristics of natural bog pools and observation periods are too short to conclude whether this will be completely possible (Iamers et al. 2002, McNeil & Waddington 2003, Tuittila et al. 2003, Cobbaert et al. 2004, Amon et al. 2005, Mazerolle et al. 2006). At least on human time-scales, impacts of disturbance in peat can be irreversible (Holden 2005).

In Europe since the nineteen-nineties, there are attempts to reinstall the biotope moor with nature-near water conditions and proportions. Such attempts are financed mainly by the European community. Currently, a European legislation to preserve moor as a biotope is lacking and regulations are left as a task of the different countries. Some moors in Europe are however protected under the FFH (Fauna, Flora, and Habitats)-directive. In Switzerland, all peatlands have been protected and no peat harvesting is possible anymore. In Germany, peat cutting is regulated in the Natural Conservation Laws. Withdrawal of peat is always connected with restoration or compensation sanctions. The situation in the Baltic States is similar (International Peat Society 2001). Nevertheless of any recreation attempts, the resource peat is finite in reserves.

The German reserves released for harvest are estimated to last maximum 25 years for the young lighter peat ("Weisstorf" in German; peat of less decomposed plant material used mainly in horticulture) and maximum 40 years for the older black peat ("Schwarztorf" in German; denser matured peat used in the past for heat and fuel even after the Second World War). Alone the commercial nurseries in Germany are consuming 6 million cubic metres of peat per year for culture substrates. A further 10 million cubic metres of peat are turned into culture substrate and marketed for commercial and private gardening inside and outside of the country (Vogtmann 2005). Currently, peat is supplied to Germany by mining the last huge upland moors in Europe, mainly in the Baltic area (Bragg & Lindsay 2003).

Replacement of peat by other suitable materials in horticulture will contribute to saving the last ancient peat reserves naturally present in unique non-readily restorable ecological biotopes (upland moors) together with rare animals and plants.

Peat substitutes

As a substrate for plant growth, peat fulfils several different functions in giving footholds to the roots, in serving in water, nutrient and mineral supply to the roots, as insulation of roots against extreme temperatures and in protection against adverse micro-organisms. Such tasks need also be accomplished by suitable peat substitutes.

Various types of farm, industrial and household wastes have been tested as peat surrogates, individually or in combination, raw or composted. Among these

were municipal waste compost, saw dust, wood chips and tree barks, coconut coir dust and the woody endocarp of other stone fruits, various types of shells and hulls, straw, spent mushroom compost, cotton gin, litter from poultry breeding, brewery sludge, olive mill waste, corrugated cardboard, paper mill sludge (sometimes referred to as "biosolids") and others (Jensen et al. 2001, Papafotiou et al. 2001, 2005, Arenas et al. 2002, Stocks et al. 2002, Clemmensen 2004, Evans & Gachukia 2004, Gariglio et al. 2004, Zhang et al. 2004, Zeytin & Baran 2004, Abad et al. 2005, Chong 2005, Urrestarazu et al. 2005). Implementation of plant growth substrate from such wastes at the same time serves the society to dispose garbage and waste in ecologically friendly ways and to conserve landfill space. However, various problems have to be overcome when using wastes for plant growth substrate production: the overall variability of the raw materials, obnoxious odours, contaminants of wastes with heavy metals, organic chemicals, plastic, glass, metal, etc., balanced concentrations of nutrients and minerals, phytotoxicity due to individual oversupplied nutrients, high salt levels, unfavourable pH values for micro-nutrient deficiencies, difference in species responses, type of plant (annual, perennial, woody), and weed growth (Chong 2005, Moore 2005).

Plant growth substrates need to have a particle structure that allows sufficient water retention but also leaves room for aeration. Preferentially, nutrients are bound to the solid particles and released to need. Mature composts that have undergone thermophilic aerobic decomposition may supply at the beginning such properties (Garcia-Gomez et al. 2003). Municipal wastes present a primary source of compost but very often, the composts contain excess salts and have a high pH (McLachlan et al. 2004, Chong 2005). Leaching prior to use can optimise the compost salt content and pH (Mazuela et al. 2005). Pure composts might therefore be used for rooting and short-time growing of horticultural crops but plants held for longer time in containers need support by other solid materials, e.g. coarse sand, perlite, pumice, and calcined clays (Bilderback et al. 2005). The biostability of the substrate material can take influence on the steady-state nutrient level in the root zone (Marfa et al. 2002) and pure compost has a lower aeration (Bugbee 2002). Moreover, individual plant species react very variable on percentages of composts present in the substrates. Some prefer less and some more compost in the substrate formulations. A compost volume of 25-50% of the total growth substrate in most instances is suitable for plant growth (Moore 2005). Composts generally provide a good biological control towards various plant diseases (Litterick et al. 2004, Noble & Coventry 2005, Raviv 2005).

Of various organic wastes, recalcitrant softwood bark, wood shavings, various types of shells or hulls, and coconut coir are characterised by good physical properties that give the plant substrate longer durability (Makas et al. 2000, Zoes et al. 2001, Chen et al. 2002, Kullmann et al. 2003, Noguera et al. 2003, Scagel 2003, Gariglio et al. 2004, Chong 2005, Bilderback et al. 2005). Spent mushroom compost from *Agaricus* cultivation is rich in nutrients and has comparable good

physical properties but also a high salt content and a high pH. Nevertheless, spent mushroom compost appears in most instances to be a good material for smaller plant containers from which excess of salts easily leaches out. Spent mushroom compost was found phytotoxic to only a few very sensitive, acid-requiring plants such as *Rhododendron* and *Azalea*. However, application depends on availability of the substrate. Often, spent mushroom compost is added as an amendment to plant growth substrate formulations (Chong 2005).

Bark and coconut coir blended with other organic or mineral components are particularly often used as peat substitutes (Chong & Lumis 2000, Hernandez-Apaolaza et al. 2005, Raviv 2005). Particle sizes and shape influences water retention and aeration in plant growth substrates (Allaire et al. 2005, Caron et al. 2005). Coir particles with diameters in the range of 0.125 to 1 mm were found to have a remarkable and highly significant impact on these physical properties of interest, in contrast to lower and larger sizes (Fornes et al. 2003, Abad et al. 2005). Particles of bark in diameters of 1-2 mm performed well (Nkongolo & Caron 1999). Of sawdust and wood shavings, the latter was found better in water retention (Allaire et al. 2005). Compared with peat, water uptake by wood fibres is faster with increasing dryness of the substrate (Gerber et al. 1999) but wood fibres show a lower water retention (Gruda & Schnitzler 2004a). In spite of this, when the substrate is moderately compressed, tomato plants can perform as in peat (Gruda & Schnitzler 2004b).

The texture of the plant growing media influences the occurrence of fungus gnats grazing on fungi and plant tissues. Coir dust medium can perform better in protection of the pest than peat-based substrates but the overall substrate composition, available nutrients and water management play also important roles (Olson et al. 2002, Meers & Cloyd 2005). In tomato production, coir (as carbonated risk husk) had an exceptional high-temperature response (Islam et al. 2002). Mycorrhization has been reported to be better in coir dust-peat mixtures than in peat alone whilst growth of some plants (*Teucrium fruticans*, *Lavendula angustifolia*) nevertheless was depressed. In contrast, mycorrhized marigold (*Tagetes patula*) grew better in coir than in peat (Linderman & Davis 2003). The type of plant and its specific needs appear very much to determine whether plant performance on coir is equal or better than on peat - in addition to the percentage of coir used in the formulations (Stamps & Evans 1997, de Krijg & Van Leeuwen 2001, Scagel 2003). C:N ratios decrease and nitrogen mobilisation and the pH increase with higher amounts of coir in the plant growth substrate. Nitrogen availability and nutrient uptake are discussed as factors for plants performing less well in coir compared to peat (Meerow 1994, Arenas et al. 2002, Scagel 2003), but also presence of phytotoxic phenols (Ma and Nichols 2004). However, lesser growth on coir in most instances does not denote that the overall plant growth is not acceptable (Wilson et al. 2001). Coir dust certainly is a suitable alternative to peat in formulations of plant growth substrates. Coir from different geographical places, from different

grade of coconut maturation, from different methods of husk processing and from different storage age vary considerably in chemical and physical properties (Evans et al. 1996, Konduru et al. 1999, Wilson et al. 2001, Abad et al. 2002, Fornes et al. 2003). For products of constant quality, these properties will have to be considered (Abad et al. 2002).

By the places of coconut production and processing, coconut is mainly used as bulking agent in plant growth substrates in tropical Asian countries, with Sri Lanka as the leading manufacturer of horticultural substrates derived from coir dust. Coir is also available in tropical America and parts of Africa where it might be used in plant growth substrates (Abad et al. 2002). In temperate regions, softwood barks (pine bark and fir bark) predominate as a peat substitute. Not all barks can be used in elevated concentration, since they may have phytotoxic effects by inherent phenolic and other toxic compounds (Ishii & Kadoya 1993, Tsakonas et al. 2005). In other cases, positive effects on plant growth by antimicrobial actions by barks have been reported (Yu et al. 1997, Yu & Komada 1999, Kofijita et al. 2006). Bark mixtures with different composts performs well in container growth of various woody plants, obviously by higher retention of nutrients (Chong 2003). Pine bark is well established with micro-nutrients. 100% milled pine bark can be used for growth of tree seedlings – only sulphur needs to be added for optimal performance (Browder et al. 2005a,b). Sorption of herbicides to the bark can be another advantage of plant substrate formulations. Slow release of herbicides reduces the overall amount of herbicides needed in weed control (Mathers 2003, Simpson et al. 2005). From other types of plant growth substrates, herbicides are easily washed out with the run off water from irrigation (Jameson et al. 2004).

Regional availability and a limited supply of uniform and consistent quality reduce the widespread usage of peat substitutes. For more than 30 years, softwood barks are established as components in growth substrates (Lunt & Clark 1959, Joiner & Conover 1967, Brown & Pokorny 1975, Cotter 1979, Cotter & McGregor 1979, Pokorny & Austin 1982). Bark availability depends on the lumber and the paper industry and the quality depends on the methods of harvest, of process and of storage of the bark. Sawdust and wood shavings as waste product from timber industry are also long considered as ingredients in plant growth substrate (Hicklenton 1982, 1983, Handreck 1993, Zoes et al. 2001, Sahin et al. 2002). Partial digested *Eucalyptus* sawdust by the basidiomycete *Volvariella volvaceae* enhanced productivity and health of wheat seedlings and onions (Rajor et al. 1996, Ramamurthy et al. 1996). However, quantities of these wastes will not satisfy the complete need for plant growth substrates.

To fully substitute peat, there is therefore an urgent need for an easily renewable substrate of consistent quality and unlimited supply at economical prices. Being widespread geographically available, trees might fulfil all these criteria. Other attempts therefore focus on chopping tree clippings and also complete trees to evaluate the performance of the wood chips as such or in composted

form in plant substrates. In a two month experiment, azalea (*Rhododendron obtusum*) and marigold (*Tagetes erecta*) had slightly lower weights on chipped loblolly pine wood than on bark, whereas japanese holly (*Ilex crenata*) did equally well on all substrates. At least for short time growth, 100% wood chips are therefore suitable as material for container substrates (Wright & Browder 2005). Composted material from tree clippings performed better in cress (*Lepidium sativum*) germination than the undamaged chips (Beauchemin et al. 1992). Composting of the recalcitrant wood chips is however a longer-lasting process. Under optimised conditions, it takes 10 month (Suzuki et al. 2004). Chopped spent hardwood logs from fruiting body production of the oyster mushroom *Lentinus edodes* was shown to improve spinach growth in greenhouses (Kimmons et al. 2003). Such ready available composted wood substrate would therefore be of much benefit. Suitable spent hardwood logs from mushroom production are a limited and local resource that possibly can serve on small scale as plant growth medium, particularly in Asian countries with higher mushroom production on logs and wood substrates (Huang & Huang 2000, Kimmons et al. 2003; see Chapter 22 of this book). However, naturally in the forests there can be much more fungal degraded wooden material ready to process for plant growth materials.

***Heterobasidion* degraded wood as peat substitute**

Within the temperate Northern forests, the root-rot *Heterobasidion annosum* (*sensu stricto*) and closely related allies are a major threat to conifers with estimated yearly losses of € 7.9 x 10⁸ (Woodward et al. 1998, Asiegbu et al. 2005). In Europe, there are the three more or less intersterile species P (*H. annosum*, *sensu stricto*), S (recently defined as *Heterobasidion parviporum*) and F (now named *Heterobasidion abietinum*) separated by their host preferences: pine, spruce and fir, respectively (Niemelä & Korhonen 1998, Hantula & Vainio 2003, Johannesson & Stenlid 2003). *H. annosum* is the most aggressive fungus attacking pine and many other conifers but also deciduous trees. The distribution of *H. annosum* covers nearly all of Europe but the coldest pine forests in the north and the driest forests in the South. *H. parviporum* is the principle species of *Picea abies* (Norway spruce) forests and it attacks *Abies sibirica* (Siberian fir). This species is mostly recorded in the Scandinavian countries and central Europe. The main host of *H. abietinum* is *Abies alba* (silver fir) but it is also found on *P. abies* and occasionally on other conifers and deciduous trees. *H. abietinum* is distributed in southern and central Europe (Korhonen et al. 1998). Isolates from northern and eastern Asian countries (Asian part of Russia, China, Japan) have mostly been identified as *H. parviporum*. The most eastern record of *H. annosum* (*sensu stricto*) is from southern Siberia (Dai et al. 2003). Furthermore in eastern and southern Asia, the non-pathogenic *Heterobasidion araucariae* and *Heterobasidion insulare* (*sensu lato* with three different intersterility groups) occur (Dai et al. 2002, Fig. 1). *H. araucariae* has also been reported from eastern Austra-

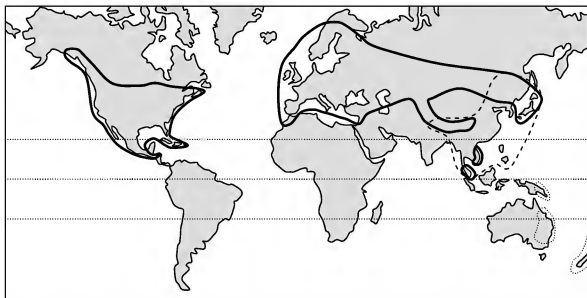


Fig. 1 Global distribution of the genus *Heterobasidion*. In the Northern parts of the world, pathogenic species of the *Heterobasidion annosum* complex are found (areas encircled in solid lines). In southeast Asia and the Australian continent, the nonpathogenic white-rots *Heterobasidion insulare* (areas encircled in dashed lines) and *Heterobasidion araucariae* (areas encircled in dotted lines) occur (after Asiegbu et al. 2005)

lia, New Zealand, New Guinea, and the Fiji Islands (Niemelä & Korhonen 1998). In North America, pathogenic P and S species are distinguished. The widely distributed North American P group mainly infests *Pinus* species. The North American S group is found in the western part of the continent on species of *Picea*, *Abies*, *Pseudotsuga*, and *Tsuga* (Korhonen et al. 1998). Taxonomically, the North American species differ from the European P and the Eurasian S groups (Dai et al. 2003, Johannesson & Stenlid 2003). Fig. 1 recapitulates the main areas of *Heterobasidion* incidences.

Large regions of Europe and of North America are affected by *Heterobasidion*. In the 1960s and 1970s, about 14% of the stems in Norway spruce plantations in Germany were affected by butt and heart rot (Kato 1967, Zycha 1976). In more recent time, in Bavaria and Saxony 10 to over 30% of all trees have been found already infected even in apparently healthy stands of younger age (up to 40 years) whilst infection rates were doubled in 70-year old stands (Rieger 1995, Schönhar 1995, Bahnweg et al. 2002). In different Norway spruce stands in Denmark, incidences at the time of felling were between 19 and 100% (Rönneberg & Jørgensen 2000). Similar broad ranges of *Heterobasidion* infestations in Norway spruce were reported from Finland and Lithuania (Tamminen 1985, Piri 1996, 2003a, Vasiliauskas & Stenlid 1998, Piri & Korhonen 2001, Vasiliauskas et al. 2002). In Finland, other species in mature conifer stands (Scots pine - *Pinus sylvestris*, lodge-

pole pine - *Pinus contorta*, Siberian larch - *Larix siberica*) suffered butt and heart rot in frequencies between 2 and 18% (Piri 2003a). *Heterobasidion* infestations were encountered in over 50% of larches (*Larix x eurolepis*) in plantations in Sweden, and in up to 70% and 29% of Sitka spruces (*Picea sitchensis*) and grand firs (*Abies grandis*), respectively (Vollbrecht et al. 1995, Rönnerberg & Vollbrecht 1999, Vollbrecht & Stenlid 1999). In Britain, decay by *Heterobasidion* was variously detected in forests in frequencies of up to 35% and more of Sitka spruces, 22% of western hemlocks (*Tsuga heterophylla*) and 7% of grand firs (Greig et al. 2001, Redfern & MacAskill 2003). In the US and in Canada, there can be similar high rates of infestation of trees by *Heterobasidion* (Goheen & Goheen 1989, Schmitt 1989, Laflamme 2005). Efforts are undertaken to eliminate the fungus from infested forest areas by silviculture management and blocking it by chemical and biological measures from entering trees (Piri 2003b, Asiegbu et al. 2005; see Chapter 14 of this book). Nevertheless, the presented examples document well that *Heterobasidion* continues to be a serious problem.

Alone in Europe, about 10% of the annually cut stem wood of *Picea* species are lost for the wood industry because of *Heterobasidion* butt and heart rot that occur within the trunks. The sum of loss represents approximately 1.7 million cubic metres of the annual *Picea* harvest (Delatour 1980). Felled stems are trimmed at the bottom from the infected timber for poor quality that, for economical reasons, will be left behind in the forests. The interior rot column can extend over several metres of stem length (Fig. 2; see also Fig. 10 in Chapter 18 in this book). In single stands, up to 37% reduction in saw timber yields in final cuttings and reduction in the sales revenues over 30% were encountered (Tamminen 1985, Kaarna-Vuorinen 2000). Trees in final stages of decay are prone to collapse and present a danger. *Heterobasidion* infected trees are susceptible to wind damage (Schmid-Haas 1994, Vollbrecht et al. 1994). In infected stands, after a storm, there can be therefore many wind-fallen trees with rotten stems of no economical value (Koch & Thomsen 2003; Fig. 2). If the infested wood from clearing stems and from fallen trees is left in the forests, this will act as source of infection for yet healthy trees (Pukkala et al. 2005). Removal from the forests will positively influence the general health and the sustainability of the forests.

Wood naturally infected with *Heterobasidion* has low marketing chances. At the time being, decayed spruce wood can only be sold under high mark-downs, if at all. An innovative idea is to use the decayed wood as raw material for planting substrates to substitute peat in horticulture. With 1.7 million and more cubic metres of rotten stems of spruce, *Heterobasidion* decayed wood can therefore make up large amounts of the volume of peat used each year in Germany for production of plant growth substrates (see above).

The value of decayed wood as a growth material is supported by observations from forests. Fallen decayed trees prepare favourable seedbeds with special tem-



Fig. 2 Views of a spruce with heart rot fallen in 1999 by the storm "Lothar" on the Dinkelberg close to Rheinfelden, south of the Black Forest (photos: Wolfgang Helle)

peratures, water regimes, nutrient composition and soil strength, well suited for example for the regeneration of various types of spruces (Anderson & Winterton 1996, Lieffers et al. 1996, Kneeshaw & Burton 1997, Ulanova 2000, Ruel & Pineau 2002). Especially in subalpine spruce stands and at dry places, natural regeneration is successful when the seedlings grow on rotten wood. Eichrodt (1969) compiled in his dissertation work advantages particularly of *Heterobasidion* decayed spruce wood for the growth of young spruces:

- higher percentage of germination
- better growth due to spontaneous development of mycorrhiza
- lower rate of mortality among the seedlings
- good physical conditions such as absence of stagnant moisture, absence of substrate hardening, lower evaporation and water storage
- favourable competition circumstances for spruce seedlings.

These advantages should also hold to plant growth substrates in horticulture. Successful application of such inferior spruce wood in agriculture and gardening through peat replacement will bring about a drastic value increase.

The Toresa® Deutschland GmbH, a company in Germany trading plant growth substrates, produces peat substitutes on the bases of wood fibres impregnated with special additives (e.g. nutrients – N, P, K, lignite for dyeing) that can replace peat even in professional cultivations. The peat substitute Toresa® is produced in the so called Retruder® process (Kharazipour 2004; Fig. 3). Two mutually mashing reversible screws within a Retruder® ("re-verse ex-truder", a further development of an extruder; Zimmermann 1994) shred wood chips as the raw material under frictional pressure, heat (80–100°C), and steam. Simultaneously, the resulting wood fibres are impregnated with additives as required. Most importantly in this special process, every wood fibre receives an equal coating of

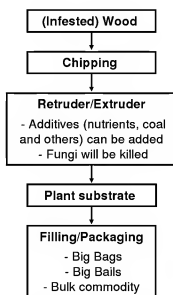


Fig. 3 Schematic diagram of the peat substitute production. The Retruder® process has originally been developed for bare wood but can also be performed with infested wood

the respective additive mixture, which will deeply impregnate into the fibre structure.

The Retruder® process can also be applied with chips from *Heterobasidion*-infested wood (Fig. 4). Compared to the compact starting material, the volume of the resulting loose fibres is four-fold increased. Due to the high operation temperature, the fungus is killed during the process of fibre disintegration. In consequence, there is no danger of distribution of an infectious fungus with produced plant substrates. After leaving the Retruder®, the new product (called plant substrate of fermented wood = PSF-Wood) is sterile. Chips from *Heterobasidion*-infested wood have two further technical advantages: the required energy for fibre



Fig. 4 Chips of *Heterobasidion*-infested spruce wood (left), peat substitute (PSF-Wood) without (middle) and with additives (right).

Table 1 Substrate mixtures tested for growth of *Carpinus betulus* (Büchner 2004)

Substrate	Formulation
1	100 % Fruhstorfer Einheitserde, Type N
2	50 % (vol.) Fruhstorfer Einheitserde, Type N 50 % (vol.) PSF-Wood
3	50 % (vol.) Fruhstorfer Einheitserde, Type N 25 % (vol.) Spruce/pine chip mixture, Retruder® processed 25 % (vol.) PSF-Wood
4	50 % (vol.) Fruhstorfer Einheitserde, Type N 16.7 % (vol.) Spruce/pine chip mixture with bark, Retruder® processed 16.7 % (vol.) Wood sticks (10 x 20 mm), Retruder® processed 16.7 % (vol.) PSF-Wood
5	100 % PSF-Wood

disintegration can be reduced by approximately 30% due to the loosened wood structure (A. Kharazipour et al., unpublished results). For the same reason, the durability of the Retruder® screws increases.

Investigations with different plant types (forest trees, vegetable plants, ornamental plants) showed that the PSF-Wood fibres are well capable of substituting peat in professional cultivations. Büchner (2004) used five different substrate mixtures (Table 1, Fig. 5) to test in green houses the growth of hornbeam (*Carpinus betulus*). Every week for 4 month, the trees were fertilised by watering ["Flory® 3 Grün 15-10-15+2" (Euflo GmbH, München, Germany) 1 ml/l water; 80 ml of this dilution per 2 l plant pot]. In shoot growth, *C. betulus* performed equally well or even better in substrates with decayed wood fibres compared to a

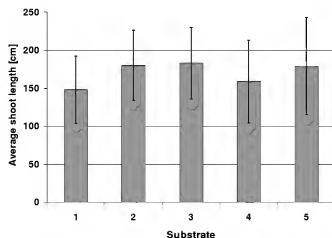


Fig. 5 Average shoot length of *Carpinus betulus* after 4 month cultivation in different substrates. For the formulations of the substrates 1 to 5, see Table 1

standard peat growth substrate (Fruhstorfer Einheitserde, Type N; Industrie-Erdenwerk Archat GmbH u. Co. KG, Lauterbach-Wallenrod, Germany), even in the case of 100% PSF-Wood (Fig. 5). Moreover, an enhanced root development was seen in the different formulations containing PSF-Wood (not shown).

Substrates with PSF-Wood function also with vegetables and ornamentals. Root growth of radish (*Raphanus sativus*) and French marigold (*T. patula* 'Sparky Mix') is enhanced in the wood fibre substrates, the shoots develop well and the ornamentals blossom plentifully (Fig. 6 and not shown). Since conifer wood is generally low in nitrogen (Hattenschwiler et al. 1996, Nordin et al. 2001, Payne 2002, Kostianinen et al. 2004, Franco et al. 2005), positive effects on growth are seen upon addition of nutrients to PSF-Wood (Fig. 6A).

All in all, our experiments with *Heterobasidion*-infested wood show that substrates with PSF-Wood have an improved texture and stability, a reduced specific gravity, and an increased porosity with the consequences of an increased air-handling capacity, an improved water management and an enhanced rooting. The water holding capacity follows better the requirements of the plants. Due to the uncovered open-pored cellulose, 150 g N can be fixed per l PSF-Wood fibre material. During plant growth, the release of nitrogen is stable over 20 days. It is slower than in other substrates due to a lower micro-organism contamination. There is no release of NH_3 through nitrification by micro-organisms (A. Kharazipour et al., unpublished results).

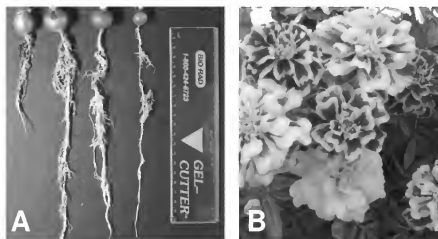
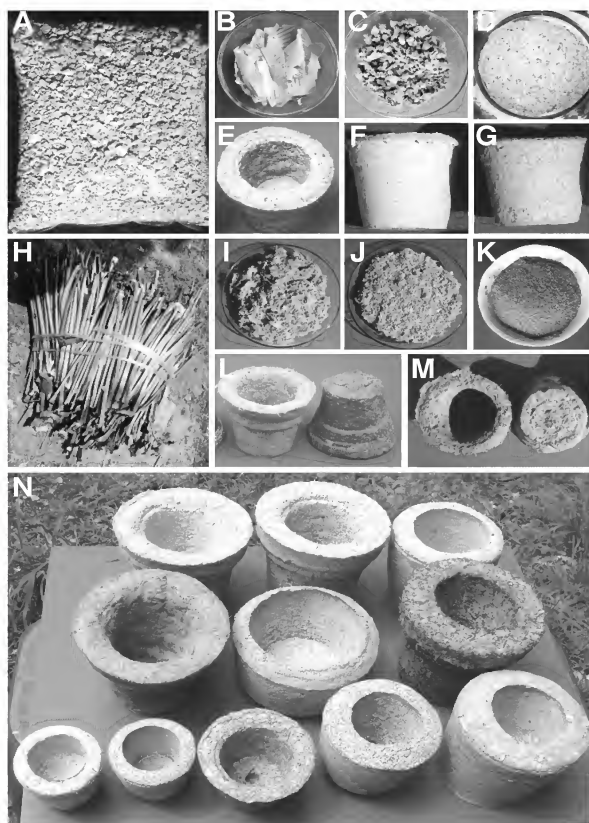


Fig. 6 A. Root development of radish plants in different substrates after 8 weeks of cultivation. From left to right: radish grown in 100% Fruhstorfer Einheitserde, radish grown in 40% vol. Fruhstorfer Einheitserde + 60% vol. PSF-Wood with additives, radish grown in 100% PSF-Wood with additives, radish grown in 100% PSF-Wood without additives. B. French Marigold (*Tagetes patula* 'Sparky Mix') cultivated in a substrate based on *Heterobasidion*-decayed wood fibres

Pot plant containers

Next to using peat as a loose planting material, peat is also employed in the commercial production of pot plant containers and of mini-pots and pellets for seed germination and rooting of shoots (Beltz 2005; <http://jiffypot.com/>). Various lignocellulosic waste materials (e.g. recycled paper pulp, saw dust, coir, agricultural wastes) are available that principally can replace peat in this function (Fig. 7 and 8). In Thailand, governmental programmes promote the development of pot plant containers from such waste materials. Recycling in form of pot plant containers can help to overcome local disposal problems of accumulating wastes. Water hyacinth, a fast growing pest that frequently needs to be removed from Thai rivers, for example presents such a local waste problem. At times when the material occurs in sufficient amounts, as an extra activity by small farmers for the own need or possibly the local market, it can be chopped and subsequently pressed into pot plant containers by simple manually-operated compactors (Fig. 9). Materials of different texture might be mixed to meet fluctuations in their availability and to

Fig. 7 Lignocellulosic materials tested in Thailand for pot plant containers. A. Dried recycled paper pulp as raw material. B. Used paper before pulping. C. Wetted recycled paper pulp before container pressing. D. Coir (7%) is mixed into the wetted recycled paper pulp giving a homogenous mass. E. Pot plant container from 100% pulp of recycled bleached paper, F. from 7% coir - 93% pulp of recycled bleached paper, and G. from 7% coir - 93% pulp of recycled unbleached paper. H. Leaf stalks from freshly harvested water hyacinth and I. dried water hyacinth fibres. J. Dried fibres from banana stalks. K. Saw dust from mixed wood. L. Pot plant container from 100% water hyacinth fibres (left) and 100% banana fibres (right). M. Pot plant containers from water hyacinth fibres mixed 1:1 with banana fibres. N. A selection of pot plant containers of different material and sizes: pulp of recycled bleached paper mixed 1:1 with banana fibre, 1:1 with coir, and 1:1 with water hyacinth fibre (upper row from left to right); 100% banana fibre, pulp of recycled bleached paper mixed 1:1 with coir, and pulp of recycled bleached paper mixed 1:1 with fresh grass (middle row from left to right); pulp of recycled bleached paper mixed 1:1 with banana fibres (lower row, outer left), 1:1 with coir (lower row the three containers in the middle) and 1:1 with water hyacinth fibres (lower row, outer right). All combinations of materials were successfully tested in serial plant growth tests with 150 to 200 containers of a height of 9 cm, an internal Ø of 11 cm and a wall thickness of approx. 1 cm (approximately 173 cm³ volume of material) and *Kalanchoe* (Flaming Katy) cuttings as test plants. For growth tests, containers were filled with a mixture of 1:1:1:1 sand, clay, bean compost, dried chicken dung and paddy husks charcoal. Up to four month, 100 ml of water per day was given to the plants (Chaisaena et al. 1998, 2001, 2002)



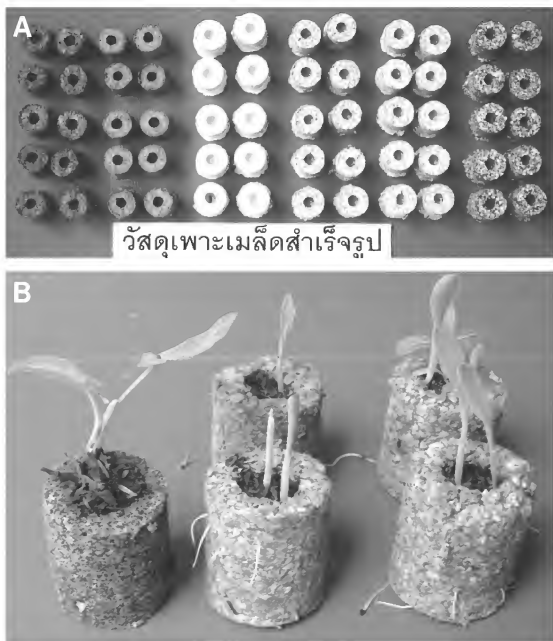


Fig. 8 A. Mini-pots of 2.5 cm in height, an outer \varnothing of 2.9 cm, a total material volume of 15.8 cm³ with an internal cavity of 1 cm in \varnothing and a depth of 1.2 cm. Materials shown in the upper photo from left to right: 100% banana fibres, 100% water hyacinth fibres, 100% of pulp of recycled bleached paper, pulp of recycled bleached paper mixed 1:1 with banana fibres, pulp of recycled bleached paper mixed 1:1 with water hyacinth fibres, and pulp of recycled bleached paper mixed 1:1 with coir. B. A young offshoot of *Syngonium* (arrowhead vine) planted for rooting into a mini-pot made from pulp of recycled bleached paper mixed 1:1 with coir is shown at the left side in the photo below. Four mini-pots made from pulp of recycled bleached paper mixed 1:1 with water hyacinth fibres in which maize kernels germinated are seen at the right side of the photo (Chaisaena, W. et al. 2003)



Fig. 9 Manually-operated compactors for the small-scale production of pot plant containers in Thailand

give optimal consistencies and stabilities (Fig. 7, 8, and 10). In manual production of pot plant containers and mini-pots, the choice of material is not as critical for the manufacturing process (Chaisaena, W. et al. 1998, 2001, 2002, 2003). For production on larger industrial scale, the consistency of the material and the available

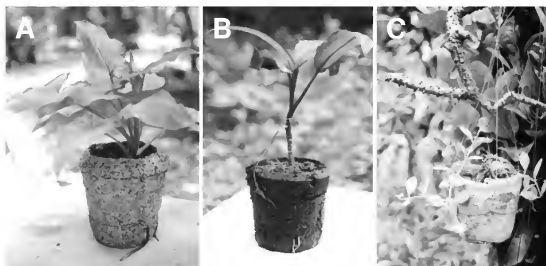


Fig. 10 Pot plant containers of 7% coir - 93% pulp of recycled bleached paper have a good porosity and therefore offer plant roots a good aeration. Upon 3-month outdoors growth of *Syngonium* sp. on a roof-protected shelf (A) and 6-month outdoors growth of *Cordyline fruticosa* (false palm; B) in soil contact on a field, aerial roots grow out of the pots. C shows the epiphytic climbing vine *Dischidia oiantha* hanging for one year outdoors under a roof and demonstrates the very strong nature of the containers (W. Chaisaena, unpublished observations)

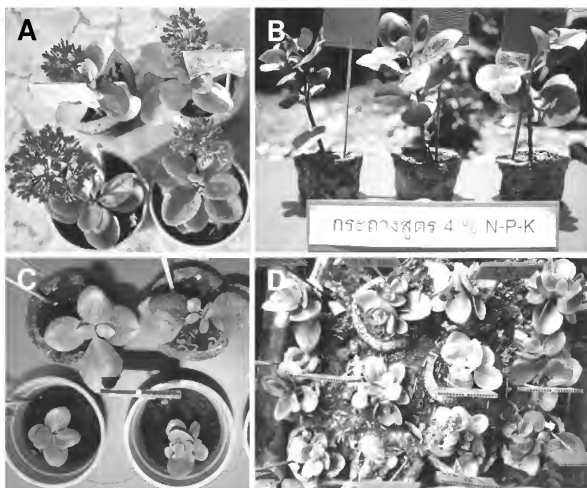


Fig. 11 Comparison of growth of *Kalanchoe* cuttings in containers of 7% coir - 93% pulp of recycled unbleached paper and in plastic containers. In a first experiment, cuttings in coir-pulp containers grew less well and leaves were light-green due to leaching of nutrients into the container (A). Upon addition of a commercial fertiliser at a final concentration of 4% N-P-K to the coir-pulp mixture used for container production, *Kalanchoe* cuttings grew well with a better height (B) and larger leaves than control plants in plastic containers (C). After cultivation for 4 month on a concrete floor, containers were embedded into soil for further growth of the plants (D). Over a period of a month, containers were slowly degraded allowing spreading of roots into the surrounding soil (Chaisaena et al. 2001, Chaisaena, P. et al. 2003)

amount will be decisive for a steady, technically unobstructed production of constant product quality. Pot plant containers from coir and from *Miscanthus* straw are offered on the European markets but with yet little market share because of a price as twice much as that of plastic pots. Depending on the consistency of the material, not all such “biopots” can be applied in sophisticated, high-throughput planting machines (Beltz 2005).

In laboratory trials, leaching of nutrients has been observed from planting substrates into the surrounding fibrous, lignocellulosic material of pot plant containers, with the effect that plants show symptoms of chlorosis. Addition of fertilisers to the lignocellulosic material of a poor nutrient condition prior to container production can avoid such problems (Fig. 11; Chaisaena et al. 2001, Chaisaena, P. et al. 2003, Beltz 2005). At an older growth age, seedlings and rooted cuttings can be implanted into soil together with the pot plant container that slowly will be degraded, allowing the roots of the plants to grow into the surrounding soil (Fig. 11D). In long-term culturing of plants in "biopots", faster degradation of the biological pot material can however be a disadvantage. Moreover, unsightly fungal and algal biofilms may form on the outside limiting the use of "biopots" for keeping plants for decoration within houses (Beltz 2005).

Conclusions

Classical plant growth substrates base on peat, which is a finite resource in human time scales difficult to renew. Fibrous lignocellulosic wastes such as coir, saw dust, wood fibres, and other plant fibre material are used as alternatives in production of plant substrates. However, the high demand for plant growth substrates requires further sources of suitable, preferentially renewable materials. Our research newly showed that *Heterobasidion*-infested wood can easily be converted into valuable plant growth substrates. In countries with large conifer forests and high *Heterobasidion* infestation, such transformed wood could therefore in the future replace at least parts of peat-based growth substrates. The amounts of *Heterobasidion*-infested wood in the Northern hemisphere are suitable for industrial production of such a product of constant quality. In tropical countries with various types and qualities of fibrous agricultural wastes and different social and economical structures, lignocellulosic wastes might be converted into plant growth substrates and pot plant containers on smaller scales, according to the local incidences of the recyclable wastes and the demand for pot plant containers and potting material (Armington 1969).

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In the year 2001, Prof. Dr. Ursula Kües was appointed at the Faculty of Forest Sciences and Forest Ecology of the Georg-August-University Göttingen to the chair Molecular Wood Biotechnology endowed by the Deutsche Bundesstiftung Umwelt (DBU). Her group studies higher fungi in basic and applied research. Research foci are on mushroom development and on fungal enzymes degrading wood and their applications in wood biotechnology.

This book has been edited to thank the DBU for all support given to the chair Molecular Wood Biotechnology. Contributions to the book are from scientists from Göttingen recognised in different fields of forestry and wood science. Chapters presented by members of the group Molecular Wood Biotechnology introduces into their areas of research. The book is designed for interested students of wood biology and wood technology but will also address scientists in the field.



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